PART 1.

A COMPARISON OF REGULATORY MECHANISMS OF LUTEINIZING HORMONE PROLACTIN AND GROWTH HORMONE EXOCYTOSIS IN PERMEABILIZED PRIMARY PITUITARY CELLS.

PART 2.

THE EFFECT OF DIVALENT CATIONS ON LUTEINIZING HORMONE AND PROLACTIN EXOCYTOSIS IN PERMEABILIZED PRIMARY PITUITARY CELLS.

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DEDICATION

To my husband John, for all his continued love and support without which this dissertation would not have been possible.

LIST OF ABBREVIATIONS USED

Ba^{2+}	:Barium
Ca^{2+}	:Calcium
free Ca^{2+}	:Free calcium concentration
cAMP	:Adenosine 3',5'-cyclic monophosphate
Cd^{2+}	:Cadmium
Co^{2+}	:Cobalt
DAG	:Diacylglycerol
EC 50	:Concentration effecting half-maximal response
EGTA	:ethylene glycol-bis(β-aminoethyl-ether)-N,N'-tetraacetic acid
GH	:Growth hormone
GHC	:Pituitary tumour cell line secreting GH
GHRH	:Growth hormone releasing hormone
GnRH	:Gonadotrophin releasing hormone
Gi	:Inhibitory heterotrimeric G protein
Gs	:Stimulatory heterotrimeric G protein
HEPES	:4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IBMX	:Isobutylmethylxanthine
InsP3	:Inositol trisphosphate
La^{3+}	:Lanthanum
LH	:Luteinizing hormone
MEM	:Minimal Essential Medium
Mg^{2+}	:Magnesium
Mn^{2+}	:Manganese
Ni^{2+}	:Nickel
NEM	:N-ethylmaleimide
OAG	:1-oleyl 2-acetyl sn-glycerol

Pb^{2+} :Lead

pCa :Negative log calcium ion concentration

PIPES :1,4-piperazinediethanesulfonic acid

PKA :Protein kinase A

PKC :Protein kinase C

PMA :Phorbol 12-myristate 13-acetate

PRL :Prolactin

SEM :Standard error of the mean

Sr^{2+} :Strontium

SS :Somatostatin

TRH :Thyrotropin-releasing hormone

VIP :Vasoactive intestinal peptide

VSCC :Voltage-sensitive calcium channels

Zn^{2+} :Zinc

TABLE OF CONTENTS

PART 1.

A COMPARISON OF REGULATORY MECHANISMS OF LUTEINIZING HORMONE, PROLACTIN AND GROWTH HORMONE EXOCYTOSIS IN PERMEABILIZED PRIMARY PITUITARY CELLS.

1.INTRODUCTION.

The hypothalamus secretes a number of substances which have a stimulatory or inhibitory effect on the release of hormones from the anterior pituitary. Gonadotropin-releasing hormone (GnRH) is a decapeptide secreted by the hypothalamus in a pulsatile manner. The release of the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), is regulated chiefly by GnRH but is also influenced by a number of other factors.

Growth hormone releasing hormone (GHRH) and somatostatin (SS) regulate the secretion of growth hormone from the somatotrophs. Thyrotropin-releasing hormone (TRH), vasoactive intestinal polypeptide (VIP) and dopamine regulate prolactin (PRL) exocytosis in the pituitary lactotroph.

Relatively little is known about intracellular signal transduction and events leading to exocytosis. When investigating intracellular signalling events, the plasma membrane poses a barrier between the external environment and the interior of the cell. The experimenter cannot directly manipulate the concentration of intracellular components. Thus the drawback of experimental research in the field of intracellular signalling has been the lack of accessability of the internal milieu of the cell.

Much has been published on studies concerning exocytosis in intact, primary cultured pituitary cells (1), (2) and on cell lines derived from pituitary tumours (3). When investigating the individual pathways involved in signal transduction and second messenger systems in intact cells, it may be difficult to interpret results because of "cross talk". " Cross talk" refers to the interactions that occur in intact cells between signalling pathways (4). Permeabilized cells offer the advantage that by regulating the components of the intracellular environment, individual aspects of these pathways can be assessed.

Exocytosis is the mechanism whereby hormones are secreted by specialised cells into their environment. These cells respond to specific stimuli by the release of proteins that have been stored in vesicles (5). These vesicles fuse with the plasma-membrane and release their contents into the external environment. Exocytosis can be described as constitutive or regulated. Constitutive exocytosis refers to the continuous secretion of proteins from the cell in an unregulated manner. Conversely, regulated exocytosis occurs only in response to specific stimuli. The present study is concerned with the intracellular events leading to regulated exocytosis in primary permeabilized pituitary cell cultures.

2.LITERATURE REVIEW.

2.1 LH EXOCYTOSIS IN INTACT CELLS.

2.1.1 THE ROLE OF Ca^{2+} AS A SECOND MESSENGER.

Catt et al.(1) have reviewed the mechanims of GnRH signalling pathways in intact pituitary gonadotrophs. The binding of GnRH agonists to high affinity receptors in the gonadotroph plasma membrane is followed by release of gonadotropins within seconds. A rapid increase in cytosolic Ca^{2+} is the major signalling event leading to gonadotropin secretion. The rise in intracellular Ca^{2+} is biphasic. The initial peak is due to the mobilization of intracellular calcium stores. Inositol trisphosphate (InsP3) is responsible for this mobilization of Ca^{2+}. LH release follows the same biphasic pattern with an initial rapid spike followed by a sustained plateau.

The slower delayed plateau of elevated Ca^{2+} is highly dependent on the entry of extracellular calcium. Endocrine cells have two types of voltage-sensitive calcium channels (VSCC), termed L and T VSCC. L "long-lasting" VSCC have the following characteristics: high conductance, a prolonged opening time, activation by large changes in membrane potential and a slow inactivation time. L VSCC are mainly involved in the regulation of intracellular Ca^{2+} and are activated by neurotransmitters, hormones, toxins and dihydropyridine agonists. They are inhibited by organic calcium channel blockers, including dihydropyridine antagonists, which are specific for L channels at low concentrations.

T "transient" VSCC, unlike L VSCC, are of low conductance, are activated by small changes in membrane potential and rapidly undergo voltage-dependent inactivation. T VSCC are not affected by low (micromolar) concentrations of organic calcium channel agonists and antagonists. These channels are probably responsible for the initiation of action potentials rather than the regulation of cytosolic calcium (1). Calcium entry through these two types of Ca^{2+} channels contributes to the sustained increase of cytosolic calcium during GnRH action. During the spike phase of LH release dihydropyridine-insensitive channels are the major route for extracellular calcium entry (1), (6). As yet the mechanism of how the GnRH receptor is linked to these calcium channels is unknown.

2.1.2 THE ROLE OF PKC IN LH EXOCYTOSIS.

The tumour-promoting phorbol esters are known to be activators of PKC (7). In 1980, Smith and Vale. (8) observed that phorbol esters stimulated LH secretion in intact pituitary gonadotropes. These observations implicated PKC in the signal transduction pathway. In chromaffin cells PKC is thought to be a mediator of Ca^{2+}- stimulated exocytosis (9). GnRH stimulates inositol phospholipid turnover resulting in increased diacylglycerol, an activator of PKC (10).

However, there have been conflicting reports in the literature as to the role of PKC in GnRH mediated LH exocytosis. McArdle et al. (11) showed that GnRH-induced LH release was unaffected in pituitary cells which had been depleted of PKC. Other studies have shown a significant impairment of LH reponse in PKC depleted cells (12).

2.1.3 THE ROLE OF cAMP IN LH EXOCYTOSIS.

Literature reports have been conflicting concerning the role of cAMP in GnRH-stimulated LH release (13), (14). Studies recently conducted on rat pituitary cell cultures concluded that cAMP was not involved in the regulation of LH exocytosis (2).

However other studies have demonstrated LH exocytosis in response to cAMP stimulation. Davidson et al. (15) observed that forskolin, an activator of adenylate cyclase, induced a large increase in LH release in ovine as well as chicken pituitary cell cultures. This data perhaps represents a species difference.

2.2 LH EXOCYTOSIS IN PERMEABILIZED CELLS.

A novel permeabilized primary pituitary cell system has been devised and implemented in our laboratory by Van der Merwe et al.(16). This model has been used in all experiments described in this study.

The plasma membrane poses a barrier to the interior of the cell. Staphyloccoccus aureus α-toxin is a protein exotoxin of Mr 34 000 Da. Monomeric α-toxin inserts into phospholipid bilayers including biological membranes. It assembles into a transmembranous, hexameric, ring-shaped protein with a central pore of diameter 2-3 nM (17). Membranes treated with α-toxin are rendered permeable to molecules with Mr 5200 Da (17). Monomeric α-toxin is too large to pass through the pore and therefore only the plasma membrane is permeabilized.

The free passage of low molecular weight molecules across the plasma membrane facilitated the regulation of the intracellular composition. The medium used to permeabilize the cells and to stimulate exocytosis resembled the physiological intracellular concentrations with respect to pH and electrolytes. The intracellular free Ca^{2+} could be maintained in the physiological range by the addition of EGTA to the medium.

By changing the composition of the stimulation media the effects of Ca^{2+}, cAMP and PKC on exocytosis on permeabilized cells were studied.

2.2.1 Ca^{2+} AND ATP REQUIREMENTS IN LH EXOCYTOSIS.

Van der Merwe et al. (16) observed that in α-toxin permeabilized cells free Ca^{2+} stimulated exocytosis half-maximally at a concentration of 2-3 μM and maximally at a concentration of 10 μM. When permeabilized pituitary cells were allowed to equilibrate with ATP-free buffer, Ca^{2+}-stimulated exocytosis decreased at a rate equivalent to that of ATP efflux. The exocytotic response to Ca^{2+} could be restored by the addition of millimolar concentrations of ATP. These findings demonstrated that there was an absolute ATP requirement in exocytosis. cAMP-stimulated and phorbol ester-stimulated exocytosis also required similar concentrations of ATP (18).

2.2.2 PKC PATHWAY /PHORBOL ESTER-STIMULATED LH EXOCYTOSIS

Using the permeabilized pituitary cell model described above, the free Ca^{2+} could be controlled. This allowed the interaction between Ca^{2+} and PKC to be studied further and evidence was obtained to show that Ca^{2+} and PKC acted by separate mechanisms (19).

The evidence was as follows. When the cells were continuously stimulated with high Ca^{2+} they became refractory to Ca^{2+} yet retained their ability to respond to phorbol ester. This implied that these pathways were able to act independently.

In order to further examine this finding PKC was inhibited whilst allowing Ca^{2+} -stimulated exocytosis. Staurosporine, a potent though non-specific inhibitor of PKC (20), inhibited phorbol ester-stimulated but not Ca^{2+}-stimulated exocytosis. Thus cells depleted of PKC could still respond to Ca^{2+}.

From these results it was concluded that Ca^{2+} and PKC had stimulatory effects on LH exocytosis, and that these two secretagogues acted by independent mechanisms.

2.2.3 ADENYLATE CYCLASE /cAMP PATHWAY.

The effects of cAMP on LH exocytosis have been studied. In ovine pituitary cells (18) cAMP was found to stimulate exocytosis at all Ca^{2+} concentrations but was markedly synergistic in the presence of phorbol ester. This synergism operated even at low Ca^{2+} concentrations (pCa 7) with half-maximal effect at a Ca^{2+} concentration of 3 μM (EC 50 = 3 μM). The synergism with phorbol ester was observed at physiological cAMP concentrations.

2.2.4 ARACHIDONIC ACID AS A SECOND MESSENGER.

Recently studies have been conducted in our laboratory on the stimulatory effects of arachidonic acid on LH exocytosis (Dr P. Kaye , manuscript in preparation). The results led to the conclusion that the effect of arachidonic acid on LH release was due to a non-specific, detergent-like effect on the cell membrane. However, Naor et al. (21) suggested that arachidonic acid exerted its effect on gonadotropin secretion after conversion to leukotrienes by 5-lipoxygenase.

2.2.5 GUANINE-NUCLEOTIDE BINDING PROTEINS- THEIR ROLE IN LH EXOCYTOSIS.

The guanine nucleotide binding proteins play an essential role in the process of signal transduction and LH secretion (22). G-proteins belong to the group of heterotrimeric proteins which are active when GTP is bound to the protein and inactive when GDP is bound.

More recently however, another class of GTP-binding proteins has been described (23). These GTP-binding proteins are thought to have a more direct effect on secretion. This group of GTP-binding proteins has a monomeric structure and are structurally related to the RAS oncogene products (24). From this group a GTP-binding protein named Ge has been postulated to be involved in regulated LH exocytosis (23).

Both stimulatory and inhibitory effects of GTP-analogues have been observed in permeabilized pituitary cells (25). The inhibitory effect is distal to second messenger generation and thereby points to involvement of an inhibitory GTP-binding protein directly in the exocytotic process.

2.3 PRL EXOCYTOSIS IN INTACT CELLS.

The hypothalamus exerts a major regulatory influence on the prolactin-producing cells of the anterior pituitary. Dopamine is the main hypothalamic prolactin inhibitory factor (26). Thyrotrophin releasing hormone (TRH) as well as vasoactive intestinal polypeptide (VIP) are known to exert stimulatory effects (26). Bjoro et al. (27) observed that TRH induced a biphasic pattern of PRL release consisting of a rapid transient initial phase followed by sustained secretion at a later phase. In contrast VIP induced a delayed monophasic response.

2.3.1 THE ROLE OF Ca^{2+}, cAMP AND InsP3 IN TRH-STIMULATED PRL RELEASE.

TRH has been shown to induce biphasic changes in intracellular free Ca^{2+}; an immediate marked elevation of PRL release was followed by a smaller plateau-like release. Schlegel et al.(28) using the quin 2 fluorescence technique, demonstrated that the first phase of the increase in cytosolic free Ca^{2+}, following TRH stimulation was due the to mobilization of intracellular Ca^{2+} stores.

In the second phase (the smaller plateau-like release), the rise in cytosolic Ca^{2+} occurred as a result of the influx of extracellular Ca^{2+} via VSCC.

Tan and co-workers (29) reported that the rise in cytosolic Ca^{2+} during TRH stimulation in GH4C1 pituitary tumour cell cultures was due to both the mobilization of intracellular stores and the influx via VSCC. These authors reported that

the influx through Ca^{2+} channels accounted for approximately 75 % of maximum TRH-induced PRL release (the first phase).

TRH was, at most, only a weak activator of adenylate cyclase. The slight increase in cAMP observed with TRH stimulation in GH cell cultures occurred after phase 1 of the TRH-induced PRL release. cAMP had no effect in the first phase of TRH-induced PRL release and only a poor effect in the second phase (30).

TRH activates phospholipase C to hydrolyse phosphatidylinositolbisphosphate to produce two second messengers, namely (1,4,5) - inositoltrisphosphate (InsP3) and diacylglycerol (DAG) (31). InsP3 increases intracellular free Ca^{2+} by causing release from non-mitochondrial stores and in this way is responsible for phase 1 (immediate marked elevation) of TRH-induced PRL release.

DAG activates PKC. PMA is a phorbol ester that activates PKC. PMA induced stimulation of PRL resembling phase 2 of TRH-induced PRL release. Therefore, PKC may be important in phase 2 of TRH-induced PRL release.

2.3.2 VIP-STIMULATED PRL RELEASE.

VIP induced a delayed monophasic response of intracellular Ca^{2+} which was preceded by a rise in cAMP. Activators of adenylate cyclase such as forskolin, also induced an increase in intracellular Ca^{2+} analogous to VIP stimulation (27). This indicates that the rise in free intracellular Ca^{2+} was secondary to cAMP. The PRL-releasing effect of VIP was inhibited by a Ca^{2+}-

free medium and Ca^{2+} channel blockers such as Co^{2+} and verapamil (32). Therefore, the rise in free intracellular Ca^{2+} is a key event in VIP-stimulated PRL exocytosis.

VIP induced activation of adenylate cyclase which resulted in an increase in cAMP prior to PRL release (30). In addition cAMP analogues stimulated PRL release from pituitary lactotrophs (33). Thefore VIP-stimulated PRL release occured by activation of the adenylate cyclase / cAMP signal transduction pathway.

2.3.3 DOPAMINE INHIBITION OF PRL RELEASE.

The inhibitory action of dopamine on PRL exocytosis has been characterized by Vallar et al. (34). Dopamine inhibited the late phase of inositol trisphosphate production stimulated by TRH. In addition dopamine inhibited the TRH activation of VSCC and therefore the influx of extracellular Ca^{2+}.

Cronin et al.(35) put forward further evidence for a role of cAMP in the PRL signal transduction pathway. Pertussis toxin is a protein exotoxin produced by the bacterium Bordetella pertussis. Pertussis toxin modifies the responsiveness of cells to a variety of hormones, with its major effect as a blocker of inhibitory hormone action (36). Pertussis toxin acts on receptor sites that are linked to the cAMP-generating system, either as stimulators or inhibitors. Pertussis toxin does not demonstrate tissue or receptor specificity. Cronin et al. (35) found that pertussis toxin decreased the capacity of bromocriptine, a dopamine agonist, to inhibit PRL release. Pertussis toxin also diminished the ability of dopamine to decrease cAMP. This suggests that the dopamine receptor is coupled to adenylate cyclase.

Vallar and Meldolesi (37) concluded in support of this that D2 dopamine receptor activation inhibited adenylate cyclase and reduced cAMP concentrations.

In GH tumour cells, VIP had no effect on concentrations of InsP3 which suggested that PKC is not involved in the VIP signal transduction pathway (38).

2.4 PRL EXOCYTOSIS IN PERMEABILIZED CELLS.

2.4.1 THE ROLE OF Ca^{2+} IN PRL EXOCYTOSIS.

Ronning and Martin (39) characterizied the secretory response to Ca^{2+} in GH3 rat pituitary cells permeabilized by electric field discharge. Ca^{2+} stimulated PRL release half-maximally at a concentration of 0.5 μM and maximally at a concentration of 3-10 μM. This Ca^{2+}-stimulated PRL release was dependent on MgATP.

2.4.2 THE PKC PATHWAY IN PRL EXOCYTOSIS.

In 1986, Ronning and Martin (40) studied the effects of protein kinase C activators on PRL release in permeabilized GH3 cells. The membrane-permeable diacyl - glycerol, 1-oleoyl 2-acetyl-sn-glycerol (OAG) and PMA, stimulated PRL secretion at low Ca^{2+} (pCa 8). At higher Ca^{2+} concentrations the effects of these agents were potentiated, with maximal potentiation occurring at pCa 6. Therefore PRL release was stimulated by a PKC - activating mechanism, the activity of which was modulated by Ca^{2+}.

2.4.3 G PROTEINS IN PRL EXOCYTOSIS.

The role of G-proteins has not been fully defined in events leading to PRL exocytosis. G-proteins which stimulate adenylate cyclase are termed stimulatory G-proteins (Gs). G-proteins which inhibit adenylate cyclase are termed inhibitory G-proteins (Gi). Stimulatory G-proteins are involved in signalling pathways leading to PRL release. VIP activates Gs directly (41).

2.5 GH EXOCYTOSIS IN INTACT CELLS.

Growth hormone (GH) is secreted by the somatotrope. Two hypothalamic peptides regulate growth hormone secretion namely, somatostatin (SS) and growth hormone releasing hormone (GHRH) but through separate receptors (42).

2.5.1 Ca^{2+} AND cAMP IN GH EXOCYTOSIS.

Growth hormone releasing hormone (GHRH) induced a rise in cAMP in the somatotrope (4), (42), (43). Forskolin and isobutylmethylxanthine (IBMX) also stimulated GH secretion and cAMP accumulation. This, thereby, implicated the cAMP-dependent pathway in GH release from the somatotrope (44).

A rise in intracellular Ca^{2+} was observed in response to GHRH stimulation (4). Thorner et al.(4) postulated that the rise in intracellular Ca^{2+} could be mediated by cAMP-dependent phosphorylation of Ca^{2+} channels, this phosphorylation being a prerequisite for Ca^{2+} channel opening. This has been demonstrated in cardiac muscle cells. cAMP activates Ca^{2+} channels in the membrane of the myocyte by phosphorylation (44). The Ca^{2+} channels in the pituitary belong to the dihydropyridine-sensitive (L-type) subclass.

The inhibitory effects of SS on GH exocytosis have been described. SS decreased the rise in intracellular Ca^{2+} in response to stimuli. The mechanism has been proposed as follows. SS inhibited the activation of adenylate cyclase and thereby prevented the phosphorylation and activation of Ca^{2+} channels (42).

2.5.2 PKC AND GH EXOCYTOSIS.

Conflicting reports have appeared in the literature as to the role of PKC in GH release (42), (43). Cronin et al. (42) demonstrated that activators of PKC such as PMA potentiated the cAMP accumulation induced by GHRH. However there was no apparent effect on GHRH potency. Thus the action of PKC was to amplify the cAMP response to GHRH (42). Conversely, Ray and Wallis (43) suggested that GHRH and PMA activated independent pathways regulating GH secretion from cultured ovine pituitary cells. SS not only inhibited GHRH-stimulated but also GHRH- and PMA-stimulated cAMP accumulation (42).

The aim of the present study was to compare the intracellular signal transduction pathways in LH, PRL and GH exocytosis. A permeabilized pituitary cell model was used for all the experiments. With this model, the individual components of the signalling pathways could be investigated by manipulating the internal milieu of the cell, whilst the exocytotic machinery remained intact.

3. EXPERIMENTAL PROCEDURES:RATIONALE AND METHODOLOGY.

3.1 PERMEABILIZATION OF PITUITARY CELLS.

The majority of studies that have been done to date on the regulation of exocytosis have been performed on intact cells. The rationale for the use of permeabilized cells is the advantage it conveys in allowing manipulation of the internal milieu of the cell as described earlier in section 2.2. Permeabilization with α-toxin allowed the passage of small molecules of Mr up to 2000 Da into the cell (17). The α-toxin itself was too large to pass through the membrane and thus only the cell membrane was permeabilized. Therefore, the exocytotic machinery remained intact.

3.2 MATERIALS.

Purified,lyophilized Staphylococcus aureus α-toxin was obtained from Dr. Sucharit Bhakdi, Institute of Medical Microbiology, Justus-Liebig University, Giesen, Germany. Aliquots of α-toxin were stored at -20°C. ^{125} I was obtained from The Radiochemical Centre (Amersham, Bucks, UK). Ovine LH, PRL and GH antisera were obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), USA. The other chemicals were obtained from Sigma Chemical Company (St.Louis, MO, U.S.A.).

3.3 CELL CULTURE SYSTEM.

Cell cutures were prepared from 6-12 month old castrated male sheep. Within 30 minutes of slaughter (Cape Town Municipal Abattoir) the pituitaries were removed from the heads and placed on ice in Minmal Essential Medium (MEM; Gibco. Paisley,Scotland) buffered with HEPES (pH 7.4) and containing amphotericin B (2.5mg/L). The anterior pituitaries were dissected free from the

posterior pituitary and the capsule. Any cartilage, bone or blood vessels were removed. The anterior pituitaries were then minced and incubated in collagenase solution;

collagenase 0.9% (w/v) 155 U/ mg (Worthington Biochemical-Corp., Freehold, NJ)

18 mg/L deoxyribonuclease (Miles Laboratories,Elkhardt,IN)

in buffer B for 2-3 hours at 37°C. Buffer B comprised of;

NaCl 137 mM; KCl 5 mM; Na_2HPO_4 0.7 mM; $CaCl_2$ 0.36 mM

HEPES 25 mM (pH 7.2)

glucose 10 mM

1% (w/v) BSA (fatty acid free, Pentex fraction V-Miles Laboratories)

Any undigested material was filtered out with nylon gauze and the suspension centrifuged at 400 x g for 10 min at 20°C. The pellet was resuspended in buffer B, centrifuged again, and then resuspended in MEM buffered with $NaHCO_3$ (1g/L), containing;

10% (v/v) fetal calf serum (Gibco, Grand Island, NY)

penicillin 60mg/L

streptomycin 100mg/L

amphotericin B 2.5 mg/L

The suspension was then drawn twice through a 19-gauge needle, filtered through nylon gauze, and dispensed at a density of $4x10^5$ cells / well into 12 - well culture plates (Nunc, Copenhagen, Denmark). All the experiments were done on cell cultures after 48 hrs incubation in 5% CO_2/ 95% air at 37°C at which time the cells were adherent to the plastic.

3.4. CELL PERMEABILIZATION AND STIMULATION.

3.4.1 WASHING.

The cell cultures were washed for a minimum of three 10 min periods at 37 °C. The buffer used was buffer I, which resembled the extracellular environment. Buffer I comprised;

NaCl 140 mM; KCl 4 mM; $MgCl_2$ 1 mM; $CaCl_2$ 1 mM

glucose 8.3 mM

HEPES 20 mM (pH 7.4)

phenol red 6mg/L

0.1% (w/v) BSA

3.4.2 PERMEABILIZATION.

After washing, the cells were permeabilized by incubation in buffer IC (0.3ml/well) containing 3μg/ml α-toxin, 6mM $MgCl_2$ and 5mM Na_2 ATP. The permeabilization period was 10 min at 37°C on a slow shaker. Buffer IC comprised;

Na propionate 140 mM; KCl 4 mM

NaPIPES 25 mM (pH 6.6)

phenol red 6mg/L

0.1% (w/v) BSA

The pH of 6.6 was selected as it allows the buffering of free Ca^{2+} in the concentration range 0.1 to 30 uM using EGTA.

In the second part of this study, the effects of the divalent cations on LH and PRL exocytosis were investigated. In these experiments, the divalent cations Zn^{2+}, Cd^{2+}, Ni^{2+} and Co^{2+} were added to the permeabilization media in equivalent concentrations to those present in the stimulation media. This allowed for a pre-incubation period before the stimulation phase.

3.4.3 MONITORING OF CELL PERMEABILIZATION.

In order to monitor the permeabilization, the rate of 2-deoxy (^{3}H) glucose efflux from cells was measured. Adherent cells were incubated for 1 h at 37°C with 2-deoxy(^{3}H) glucose (0.4 μCi/ml)in glucose-free buffer I (0.75 mls/well). Cells were then washed 7 times (3 times briefly and 4 times for 5 min on a slowly rotating shaker) in buffer I (1 ml/well). The remaining radioactivity represented intracellularly trapped , membrane-impermeant phosphorylated metabolites of 2-deoxy (^{3} H) glucose. The buffer was then replaced with buffer IC (1 ml/well) containing EGTA (0.5 mM), MgCl2 (1 mmol) and α-toxin in a range of concentrations from 0 to 30 ug/ml. The radioactivity in the medium was counted on a Beckman LS 3801 liquid scintillation counter after the addition of scintillation fluid (Instagel, Packard). Total cellular radioactivity was determined after lysing the cells with 0.5 % (w/v) sodium dodecyl sulphate.

3.5 STIMULATION.

After permeabilization, the permeabilization buffer was aspirated and replaced with stimulation buffer. Stimulation buffer comprised buffer IC, pH 6.6 with $MgCl_2$ (6mM) and Na_2ATP (5mM). In addition, depending on the experiment being conducted, there were other components of the stimulation buffer as indicated in the figure legends. Stimulation was initiated by adding 0.6 ml of the stimulation media at 37°C after aspiration of the permeabilization media. The incubation time unless otherwise indicated was 20 min at 37°C. At the end of the stimulation period, the stimulation media were collected. Detached cells were removed from the stimulation medium by

centrifugation (400 x g, 10 min, 4°C). The supernatant was then poured into Eppendorf tubes for storage at -20°C. The released hormones were then measured by radioimmunoassay and for all experiments the amount of released hormone was expressed as a percentage of the total cellular hormone at the beginning of the stimulation period. (See Section 3.6.3.)

3.5.1 Ca-EGTA BUFFERS.

Van der Merwe et al. (16) have described that nominally Ca^{2+}- free buffers did not stimulate exocytosis in α-toxin permeabilized cells, despite the absence of EGTA and the presence of 1-10 μM contaminating Ca^{2+}. This can be explained by the fact that micromolar Ca^{2+} must be strongly buffered with EGTA in order to stimulate exocytosis in a-toxin permeabilized cells due to the slow rate of diffusion through α-toxin pores (16). In the absence of strong EGTA buffering, the cells are able to pump out or sequester the small amount of Ca^{2+} which enters the cell.

The free Ca^{2+} concentrations indicated in each experiment were obtained using EGTA buffers with varying concentrations of Ca^{2+} and EGTA. The required ratios of Ca:EGTA were calculated with the use of a computer program. The program was written at the University of Cape Town using the equations from Fabiato and Fabiato (45).

The Ca-EGTA buffers of varying Ca:EGTA ratios were prepared by mixing a stock solution of pure EGTA with a stock solution Ca-EGTA which had a Ca^{2+} to EGTA ratio of exactly 1:1 {Ca:EGTA (1:1)}. Both stocks were prepared from the same batch of EGTA so they had an identical EGTA concentrations (160 mM assuming 97% EGTA purity). By accurate mixing of these stocks in

different ratios (Table 1) the free Ca^{2+} concentrations required could be obtained. The pH of each stock was adjusted to 6.6, ensuring that the pH of the final Ca-EGTA buffer was also 6.6. Ca-EGTA buffers were used at a final concentration of 30 mM (EGTA) in the stimulation media. This concentration was previously shown to be optimal by Van der Merwe et al.(16) and Macrae et al.(17). Ca-EGTA buffers were used in high concentrations in order to achieve the required buffering capacity. At Ca-EGTA concentrations of less than 20 mM the buffering capacity was inadequate. Since the pH has a significant effect on the free Ca^{2+} at a particular Ca^{2+} : EGTA ratio it was essential that the pH was not perturbed from pH 6.6 and therefore all stock solutions (e.g. Na_2ATP) were prepared at pH 6.6.

3.5.2 STIMULATION WITH Mn^{2+}.

The permeabilized cells were stimulated with Mn^{2+} (chloride salt) in buffer IC containing 6mM $MgCl_2$ and 5mM ATP (sodium salt). When Mn^{2+} was used as a stimulus, EGTA was omitted from the permeabilization and stimulation steps. EGTA has a high affinity for Mn^{2+}. At the desired concentration range of free Mn^{2+}, EGTA would not be able to provide the required buffering capacity.

In the steps where EGTA was omitted, the contaminating free Ca^{2+} in the intracellular buffer ranged from pCa 6 to pCa 5. Both Mg^{2+} and Mn^{2+} bind to ATP, therefore the free Mn^{2+} concentrations were calculated using equations for multiple equilibria as described by Fabiato (46) with stability constants as given by Martell and Sillen (47). For comparison, intact cells were also stimulated with Mn^{2+} in buffer I.

3.5.3 EXPERIMENTS WITH Cd^{2+}, Zn^{2+}, Co^{2+} AND Ni^{2+}.

Cd^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+} were added to the permeabilization media in the same concentrations that were present in the stimulation media. Permeabilized cells were stimulated with Ca^{2+}, Ba^{2+} Mn^{2+} (as chloride salts),and PMA and cAMP in buffer IC. Buffer IC contained 6 mM $MgCl_2$ and 5 mM ATP (disodium salt). The intracellular buffers contained between 1 and 10 μM Ca^{2+} as determined by Ca^{2+} electrode (Radiometer type). The Ca^{2+} that was present in the buffers occurred as a result of the contamination that is commonly associated with the salts used in preparation of the buffers. EGTA was not included in the buffers to avoid chelation of the cations.

3.6 RADIOIMMUNOASSAYS.

The stimulation media were assayed for LH, PRL and GH. The antisera were obtained from the National Institute of Diabetes and Digestive and Kidney Diseases as well as the purified ovine LH, PRL and GH.

3.6.1 IODINATION PROCEDURE.

LH, PRL and GH were iodinated as follows; 3 μg of the dry peptide was dissolved in 20 ul of 0.5 M NaH_2PO4 pH 7.6 and placed in an Eppendorf tube. Chloramine T, 10 μl (25 mg / 5 ml 0.5 M NaH_2PO4 pH 7.6), together with ^{125} I 10 μl (1 μCi) was added to the tube allowing the oxidation of the peptide. The reaction was allowed to proceed for 1 min at room temperature, after which the reaction was stopped with the addition of sodium metabisulphite 50 μl (6.7mg /10 ml NaH_2PO4). The reaction mixture was then applied to a Sephadex G-50 (Pharmacia, Uppsala, Sweden.) column to separate the free ^{125} I from the iodinated peptide. The

components were eluted using barbitone buffer (0.7 M at pH 7.5) and 1 ml fractions were collected. A Crystal gamma-counter (Packard, United Technologies) was used to determine the radioactivities. The radioactivity profile showed two clearly defined peaks. The first peak consisted of fractions containing the iodinated peptide. The second peak consisted of the free unbound ^{125} I.The iodinated peptide was then aliquoted and stored at -70°C until required for use.

3.6.2 ASSAY PROCEDURES.

The samples were thawed by placing them in a 37°C incubator for 10 min. Samples were diluted as necessary using assay buffer. The assay buffer consisted of NaPIPES, 25 mM pH 6.6; NaCl 0.15 M ; EDTA (di- sodium salt), 0.01 M; phenol red 6 mg/L; NaN_3, 5 g/L; gelatin, 1 g/L; Triton X-100 0.6 ml/L. The final volume of diluted sample used in the radioimmunoassay was 300 µl. The antisera were used at a final titre of 1:2 000 000. 100 µl of antiserum and 100 µl of iodinated peptide (20 000 CPM /100 µl) was added to give a final volume of 500 ul. The assay was allowed to incubate at 4°C for 48 hours.

The antiserum-bound peptide was seperated from the unbound peptide by the addition of a 100 µl of second antibody attached to cellulose (Sac-Cel RD 70, Wellcome Reagents Limited, Beckenham, England). The assay was incubated at room temperature for 30 min after which the reaction was stopped by dilution with with 3 ml of distilled water. The bound and free radioactivities were seperated by centrifugation and the supernatant containing the free radioactive fraction was discarded.

After iodination, the proportion of radioactivity in the ^{125}I peptide preparation which bound the peptide antiserum was typically 40-50 % and was calculated as below;

Bo/T x 100

Bo = radiolabel which bound the peptide antiserum in the absence of unlabelled peptide

T = total radiolabel

Non-specific binding (NSB) was always less than 10 percent.

N/T x 100

N = radioactivity which remained in the tube in the absence of antiserum

A standard curve was obtained by using a series of concentrations of unlabelled ovine peptide of which the concentration of each peptide was known. Eight standards were used in the LH and GH assays, and ten standards were used in the PRL assay. All standards, NSB , Bo , T and samples were assayed in duplicate. The standard curve was fitted by the logistic method. Readings were only taken from the useful part of the assay which is defined as that part of the curve which lies between 80% and 20 % where ;

B/Bo x 100

B = antiserum - bound radioactivity in the presence of unlabeled peptide.

Intraassay and interassay coefficients of variation were less than 10 % in both cases.

3.6.3 TOTAL CELLULAR HORMONE CONTENT.

The hormone content of the first two wells on each cell culture plate represented basal exocytosis since no secretogogue was added to the stimulation media. The total cellular content of each polypeptide hormone was measured by solubilizing the cells of these basal wells at the end of the stimulation period and after the stimulation media had been aspirated. 0.6 ml of Triton X-100 was added to the basal wells on each cell culture plate.

The plates were then placed in a 37°C incubator on a shaker for a minimum period after 1 hr to allow solubilization of hormone. Triton X-100 has a detergent action on plasma membranes and rapidly disrupts the integrity of the cells. The time period of 1 hr was chosen for convenience as a shorter time would have sufficed. After 1 hr, the Triton X-100 was aspirated and stored at -20°C until the radioimmunoassays were performed. The peptide content of the cellular lysates was used to express the amount of hormone released (basal or stimulated) as a percentage of the total cellular content of the unstimulated cells. All data has been expressed in this manner.

4. DATA PRESENTATION.

The data points and error bars represented graphically show the mean and range of duplicate cell culture wells. Medium from each well was assayed in duplicate. Each experiment was performed on three seperate occasions in order to show reproducibility. The data shown represents the optimum duplicates of one of the three experiments.

5. RESULTS OF LH EXPERIMENTS.

5.1 MONITORING OF CELL PERMEABILIZATION.

In intact cells, the phosphorylated intermediates of 2-deoxy (^{3}H) glucose are released from the cell at a very slow rate. After permeabilization with α-toxin, 80 % of the 2-deoxy(^{3}H) glucose metabolites were released within 20 min (Fig.s 1A and 1B). Previously, it has been shown that the concentration of α-toxin to greater than 3μg / ml had no significant effect (16). Therefore in all permeabilization procedures, α-toxin was used at a concentration of 3 μg/ml.

5.2 TIME COURSE OF LH EXOCYTOSIS.

In order to determine the optimal time period to be used for hormone release studies, it was necessary to characterize the time course of hormone release in response to the secretogogues used.

LH release stimulated by free Ca^{2+}, PMA or PMA and cAMP was maximal within the first 5 min and then declined (Fig. 2). Thereafter the rate of LH release declined rapidly and reached a steady state within 15 min after stimulation. For this reason a stimulation period of 20 min was used in all subsequent experiments.

5.3 THE EFFECTS OF FREE Ca^{2+} ON LH EXOCYTOSIS.

Micromolar free Ca^{2+} stimulated LH exocytosis . LH release increased at higher free Ca^{2+} with maximal LH release occuring at 10 μM and half-maximal stimulation occuring at 2-3 μM free Ca^{2+} (Fig. 3).

5.4 THE EFFECTS OF cAMP ON LH EXOCYTOSIS.

By supplying cAMP to the cell and by allowing its entry into the cytoplasm via α-toxin pores, its involvement in LH exocytosis could be studied. Maximal concentrations of cAMP were used (30 μM). cAMP enhanced basal as well as Ca^{2+}-stimulated LH exocytosis (Fig. 3). However, the enhancement of LH release with cAMP was slight compared to that observed with PMA.

5.5 THE EFFECTS OF PMA ON LH EXOCYTOSIS.

The PKC-activating phorbol ester PMA stimulated exocytosis even at low intracellular free Ca^{2+} (pCa 8). The increase over basal LH release was several fold and further stimulation of LH exocytosis occurred with increasing free Ca^{2+}. Maximal LH release occurred at pCa 5 and a slight decrease of LH exocytosis was observed at pCa 4.5 (Fig. 3).

5.6 cAMP DOSE-RESPONSE CURVE.

A cAMP dose-response experiment was performed using concentrations of cAMP between 0 and 30 μM. Cells were stimulated both in the presence of 100 nM PMA and in the absence of PMA. At this dose of PMA synergism was maximal at 10 μM cAMP (Fig. 4).

5.7 THE EFFECT OF cAMP AND PMA ON LH EXOCYTOSIS.

When maximal effective doses of cAMP (30 μM) and PMA (100 nM) were added simultaneously to the stimulation media a marked synergistic effect was observed. The LH release at low free Ca^{2+} (pCa 8) and at higher free Ca^{2+} (pCa 5) was markedly enhanced. Maximal LH release was observed at pCa 5 with EC 50 of between

pCa 6 and pCa 5.5. At higher free Ca^{2+} (pCa 4.5) there is the same slight decrease in LH release that was consistently observed with basal as well as stimulated LH release (Fig. 3).

6. RESULTS OF PRL EXPERIMENTS.

6.1 THE EFFECTS OF Ca^{2+} ON PRL EXOCYTOSIS.

At low free Ca^{2+}, (pCa 8), PRL release was negligible. Only at higher free Ca^{2+}, (pCa 6) was PRL release found to be significantly increased over the basal release. PRL release was half-maximal at pCa 5.5 and maximal at pCa 5 (Fig. 5).

6.2 THE EFFECT OF cAMP ON PRL EXOCYTOSIS.

cAMP alone had no effect on PRL release at low free Ca^{2+} (Fig. 5). At pCa 6, there was a slight increase in PRL release which was a consistent finding in three experiments done on separate occasions. This mild enhancement of PRL release in the presence of cAMP also occured at pCa 5.5 with maximal PRL release occuring at pCa 5.5.

6.3 THE EFFECT OF PMA ON PRL EXOCYTOSIS.

In a manner similar to its effect on LH release, PMA stimulated release of PRL at low free Ca^{2+} (pCa 8). Exocytosis was further enhanced at higher free Ca^{2+} with maximal release occuring at pCa 5. At pCa 5.5, PMA-stimulated LH release was markedly enhanced over Ca^{2+}-stimulated LH release (Fig. 5).

6.4 EFFECTS OF PMA / cAMP ON BASAL AND Ca^{2+}-STIMULATED PRL RELEASE.

At pCa 8, cAMP and PMA had a marked effect on PRL release. There was approximately a 3-fold increase over PMA-stimulated PRL release at pCa 8. High free Ca^{2+} further stimulated PRL release with maximal release occuring at pCa 6. Although the effect of PMA and cAMP on PRL exocytosis was not as great as that seen with LH release, the effect with PRL could still be described as synergistic. Both basal as well as PMA- and cAMP-stimulated exocytosis showed enhancement in reponse to high free Ca^{2+} (Fig. 5).

7 RESULTS OF GH EXPERIMENTS.

7.1 THE EFFECT OF FREE Ca^{2+} ON GH EXOCYTOSIS.

GH secretion was increased in response to high free Ca^{2+}. Maximal release occurred at pCa 4.5 with half-maximal release observed between pCa 5.5 and pCa 5 (Fig. 6).

7.2 EFFECT OF cAMP ON GH EXOCYTOSIS.

cAMP had no effect on basal or Ca^{2+}-stimulated GH release (Fig. 6).

7.3 EFFECT OF PMA ON GH EXOCYTOSIS.

PMA had no effect on basal GH release. At pCa 6 PMA enhanced Ca^{2+}- stimulated GH release. There was a marked effect at higher free Ca^{2+} with maximal GH release observed at pCa 5 (Fig. 6).

7.4 EFFECT OF PMA AND cAMP ON GH EXOCYTOSIS.

Although cAMP had no effect on basal or Ca^{2+} -stimulated GH release, in combination with PMA, a synergistic effect was consistently observed (experiments were done on three separate occasions). This effect was manifest at pCa 6 and maximal GH release occured at pCa 4.5 (Fig. 6).

8. DISCUSSION.

Calcium has long been studied as the major signalling event in exocytosis. The results from this study showed that free Ca^{2+} at pCa 5 stimulated LH exocytosis with half-maximal release observed between pCa 5.5 and pCa 5. There was an inhibitory effect on LH exocytosis at higher free Ca^{2+} (pCa 4.5). PRL and GH secretion demonstrated the same response to free Ca^{2+}. The maximal effect occurred at pCa 5 and there was no stimulation observed at lower free Ca^{2+} (pCa 8 or pCa 7). The absolute amounts of hormone released as a percentage of total cellular content differed. These differences were observed between the replicate experiments for each hormone. There were also differences between the absolute amounts of the individual hormones released. This may have been the result of variation in the density of populations of cells in the cell culture plates.

Thus the data from this study indicate that free Ca^{2+} is a major second messenger system for LH, PRL and GH exocytosis.

The role of cAMP in events leading to exocytosis was studied using the permeabilized cell model. cAMP had a small stimulatory effect on basal as well as free Ca^{2+}-stimulated LH exocytosis. The free Ca^{2+} sensitivity was not altered in the presence of cAMP The enhancement of LH release by cAMP occurred when the intracellular free Ca^{2+} was strongly buffered by EGTA, thereby precluding any effect of cAMP via Ca^{2+}. These direct effects of cAMP, independent of Ca^{2+}, could be demonstrated only in permeabilized pituitary cells and not in intact pituitary cells cultures. As the free Ca^{2+} increased, a synergistic effect was observed in the presence of cAMP.

In intact cardiac muscle cells in culture, cAMP caused a rise in intracellular free Ca^{2+} (44). In these cells, β-adrenergic agonists increased the intracellular free Ca^{2+} by phosphorylation of Ca^{2+} channels (44).

In the present study, cAMP synergistically enhanced PMA- and Ca^{2+} -stimulated LH exocytosis. These findings suggest that cAMP also plays a major role in LH exocytosis, through its synergistic interactions with PKC and Ca^{2+}.

The PKC activator PMA stimulated LH exocytosis over a range of Ca^{2+} concentrations. LH exocytosis was observed at pCa 8 indicating that the PKC mechanism was operative at low free Ca^{2+} concentrations. These results are in agreement with those of Van der Merwe et al (19) who showed that free Ca^{2+} and PMA stimulated LH exocytosis by different mechanisms.

In the present study, LH exocytosis has been compared to PRL and GH exocytosis. These results show that stimulation of PRL and GH exocytosis by free Ca^{2+} is a major signalling event as has been documented for LH exocytosis. Maximal release of LH, PRL and GH occured at pCa 5 although the amount of hormone released, when expressed as a percentage of the total, varied between the three hormones.

cAMP enhanced LH secretion at low free Ca^{2+} although this enhancement was small. PRL secretion was also enhanced by cAMP but this only occurred at higher free Ca^{2+} (pCa 6). In contrast GH was not stimulated by cAMP even at maximal free Ca^{2+} (pCa 5). These findings suggest that GH release is not due to activation of the adenylate cyclase / cAMP pathway even in the presence of high intracellular free Ca^{2+}.

The phorbol ester PMA had a marked effect on PRL exocytosis at low free Ca^{2+}. This resembled the response of LH exocytosis to PMA. This observation suggested that like LH, PRL secretion occurred via activation of PKC. GH, unlike LH and PRL, was unaffected by PMA at low free Ca^{2+}, but showed a marked response to PMA at pCa 6.

LH and PRL showed a synergistic response to cAMP in the presence of PMA over a range of free Ca^{2+} concentrations. There was a striking lack of response of GH to these two secretagogues at low free Ca^{2+}. However, as the free Ca^{2+} increased, a synergistic response was observed at pCa 6.

9. CONCLUSION.

In conclusion, differences are observed in the second messenger pathways involved in LH, PRL and GH secretion. LH exocytosis utilizes three independent second messenger pathways for signal transduction. These are free Ca^{2+}, adenylate cyclase and protein kinase C pathways.

With regard to PRL, free Ca^{2+} regulates exocytosis independently of adenylate cyclase and protein kinase C. These observations for PRL are in agreement with Ronning and Martin who concluded that (in permeabilized GH3 pituitary tumour cell cultures) free Ca^{2+} regulated PRL release (39).

The results from the present study showed that cAMP regulation of PRL exocytosis was dependent on the intracellular free Ca^{2+} concentration. In the presence of PMA, the response to cAMP was enhanced even at low free Ca^{2+}. Thus cAMP is important in the signal transduction process leading to PRL release and has a vital role in its synergism with PKC.

Activators of PKC, such as PMA, stimulated PRL release at low free Ca^{2+}. The effect of PMA was potentiated at higher free Ca^{2+}. Therefore, PKC regulates PRL exocytosis independently of free Ca^{2+}. The earlier studies by Ronning and Martin (40) showed similar results for PRL release in response to PMA stimulation.

A rise in the intracellular free Ca^{2+} is a major stimulus for GH secretion. cAMP regulation of GH exocytosis occurs in the presence of a high intracellular free Ca^{2+} as well as maximal effective concentrations of PMA. The simultaneous stimulation of GH secretion with cAMP and PMA was synergistic at higher Ca^{2+}. This synergism has previously been documented in the literature

(42). Thus the regulation of GH exocytosis by cAMP is dependent on the intracellular free Ca^{2+} and the activation of PKC. The regulation of GH exocytosis by PKC is dependent on the intracellular free Ca^{2+}.

It is still unclear which G-proteins are involved in LH, PRL and GH exocytosis and further studies are required to finally define the intricate process of signal transduction and events leading to exocytosis in specialized endocrine cells.

PART 2. THE EFFECT OF THE DIVALENT CATIONS ON LH AND PRL EXOCYTOSIS IN PERMEABILIZED PRIMARY PITUITARY CELL.

10. INTRODUCTION.

The second part of this study is concerned with the effects of the divalent cations Mn^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+} and Co^{2+} on luteinizing hormone and prolactin exocytosis in permeabilized primary pituitary cell cultures. The study has been further divided into two sections. The first section examines the effects of Mn^{2+}, while the second section addresses the effects of the other divalent cations on exocytosis.

11. STIMULATION OF EXOCYTOSIS BY Mn^{2+}.

In the earlier part of this study, the signal transduction pathways regulating pituitary hormone exocytosis were examined under various conditions. Free Ca^{2+} was found to be a key factor in LH, PRL and GH exocytosis. Ba^{2+} has been shown to mimick Ca^{2+} in stimulating regulated exocytosis in certain cell types (48). Likewise, Sr^{2+} has also been reported to substitute for Ca^{2+} (48). Little has been reported in the literature concerning the effects of other divalent cations on regulated exocytosis. Previous studies on the effects of Mn^{2+} on exocytosis were conducted in intact cell systems which had been derived from a variety of cells such as pancreatic cells (49), (50), (51). Only a single study Knight et al.(52) is reported in the literature on the effect of Mn^{2+} on exocytosis in permeabilized cells. Knight and co-workers observed that Mn^{2+} was a poor stimulator of exocytosis in electropermeabilized chromaffin cells when compared to Ca^{2+}.

The present study assesses the effect of Mn^{2+} on LH and PRL exocytosis in a-toxin permeabilized cells. For methodology see section 3.5.

11.1 THE EFFECT OF Mn^{2+} ON LH EXOCYTOSIS.

Mn^{2+} stimulated LH exocytosis in permeabilized cells with maximal release occuring at 0.5 mM total Mn^{2+} (Fig. 7). At higher Mn^{2+} (10 mM) an inhibitory effect was observed.The results are representative of two previous experiments.

Mn^{2+} had no effect on LH exocytosis in intact cells at any of the concentrations of Mn^{2+} (Data not shown -results of work done by Dr.J.Davidson in our laboratory).

11.2 THE DEPENDENCE ON ATP OF Mn^{2+}-STIMULATED LH EXOCYTOSIS.

Cells were depleted of ATP after permeabilization by allowing them to equilibrate with an ATP- free buffer on ice for 40 min. The ATP leaked out of the cells during this period and thus the cells became depleted of ATP. Cytosolic ATP stores could be repleted by adding MgATP to the stimulation media.

Mn^{2+} was added to the stimulation buffer in a range of concentrations from 0 to 6 mM total Mn^{2+} in the presence or absence of ATP. Mn^{2+}-stimulated LH release was ATP-dependent (Fig. 8). Ca^{2+}-, PMA- and PMA / cAMP- stimulated LH exocytosis was also shown to be ATP-dependent (Fig. 9).

11.3 THE EFFECT OF Mn^{2+} ON PRL EXOCYTOSIS.

Mn^{2+} stimulated PRL release in permeabilized cells in a manner similar to LH release, with maximal PRL release occuring at 0.5 mM total Mn^{2+}. At higher concentrations Mn^{2+} was found to have an inhibitory effect (Fig. 10).

12 DISCUSSION.

In intact cells Mn^{2+} exerts both stimulatory and inhibitory effects on exocytosis. The mechanism which probably accounts for its inhibitory effect is the blockade of Ca^{2+} channels (53), (54), (55). Only one report has been published on Mn^{2+}-stimulated exocytosis in permeabilized cells (52).In that study, Knight et al. (52) observed that Mn^{2+} stimulated catecholamine release in electropermeabilized bovine chromaffin cells. Maximal release occurred at 0.8 mM Mn^{2+} and an inhibitory effect was observed at higher cation concentration (1 mM). These effects of Mn^{2+} are not unlike those observed in the present study. However, Knight et al. (52) reported that Mn^{2+} had a weak stimulatory effect, in comparison with Ca^{2+}. This contrasted with the strong stimulation reported in this study in pituitary cells and may reflect either a difference between the two cell types, or a difference in permeabilization methodology. The results of the present study indicated that the effect of Mn^{2+} on LH release was dependent on ATP. A similar ATP-dependence was shown for PRL in our laboratory by Dr.J.Davidson (unpublished data).

Ca^{2+}-, PMA- and cAMP- stimulated exocytosis act through ATP-dependent pathways. In other experiments performed in our laboratory, Mn^{2+} - stimulated exocytosis of LH and PRL was shown to be sensitive to inhibition by the alkylating agent N-ethylmaleimide (NEM). Hormone release due to non-specific membrane damage is insensitive to NEM.

These findings indicate that Mn^{2+} -stimulated hormone release is an energy-dependent process and is due to true exocytosis, and not attributable to a toxic effect on the plasma cell membrane.

The precise role of Mn^{2+} in the chain of events leading to exocytosis is unclear. It has been reported that Mn^{2+} activates protein kinases (56) and phosphatases (57), thus, it is conceivable that the intracellular action of Mn^{2+} occurs via phosphorylation and dephosphorylation of proteins in the signal transduction pathway.

13. THE EFFECTS OF DIVALENT CATIONS (Cd^{2+}; Zn^{2+}; Co^{2+} AND Ni^{2+}) ON LH EXOCYTOSIS AND PRL EXOCYTOSIS IN PERMEABILIZED PRIMARY PITUITARY CELLS.

13.1 INTRODUCTION.

Free Ca^{2+} has been shown to be a major stimulus in events leading to exocytosis. The rise in intracellular free Ca^{2+} may be due to the entry of extracellular Ca^{2+} through Ca^{2+} channels in the plasma membrane, or the release of intracellular stores of Ca^{2+}.

Other divalent cations have been assessed for their effects on hormone secretion. Previous reports in the literature found that the inhibitory effects of the divalent cations were mainly due to their Ca^{2+} channel-blocking activity (53), (54), (55). In this section, the effects of Cd^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+} on LH and PRL exocytosis were investigated. With the permeabilized cell model used in this study, effects other than those on Ca^{2+} channel activity could be assessed for methodology see section 3.5.3.

14 RESULTS OF DIVALENT CATION EXPERIMENTS.

14.1 THE EFFECT OF Cd^{2+} ON LH AND PRL RELEASE.

In order to first determine the optimal stimulatory concentration of Ca^{2+} and Ba^{2+} in the unbuffered system, dose-response curves for $CaCl_2$ and $BaCl_2$ were assessed (Fig. 11).

All the data presented from this section of the study is normalized as follows. 100% LH and PRL release represented maximal stimulated exocytosis in the absence of the inhibitory cations. LH and PRL exocytosis was stimulated by $CaCl_2$ 2 mM; $BaCl_2$ 2 mM; $MnCl_2$ 2 mM and maximally effective doses of PMA (100 nm) and cAMP (30 μM).

Cd^{2+} was half-maximally effective at 30 μM, with complete inhibition of stimulated LH release occuring at 100 μM Cd^{2+}. A similar inhibitory effect was observed with PRL (Figs. 12 and 13).

14.2 THE EFFECT OF Zn^{2+} ON LH AND PRL RELEASE.

Stimulated LH release was also inhibited by Zn^{2+} with half-maximal inhibitory concentration of 60 μM. Stimulated exocytosis was almost completely abolished at 100 μM Zn^{2+} (Fig. 14).

Stimulated PRL exocytosis was inhibited by Zn^{2+}, the half-maximal inhibitory concentration being 60 μM, and maximal inhibition occuring at 100 μM Zn^{2+} (Fig.15).

14.3 THE EFFECT OF Co^{2+} ON LH AND PRL RELEASE.

Co^{2+} inhibited Ba^{2+} -, Ca^{2+} -, Mn^{2+} - and PMA / cAMP-stimulated LH release. The half-maximal inhibitory concentration was 100 μM. There was complete inhibition of stimulated release at a concentration of 1 mM Co^{2+} (Fig. 16).

Co^{2+} inhibited stimulated PRL exocytosis in a manner similar to that observed with LH. The half-maximal inhibitory concentration was 100 μM and maximal inhibition occurred at 1 mM (Fig. 17).

14.4 THE EFFECT OF Ni^{2+} ON LH AND PRL RELEASE.

Ni^{2+} inhibited stimulated LH and PRL release in a manner that was analogous to the inhibition observed with Co^{2+}. The half-maximal inhibitory concentration was 100 μM with maximal inhibition observed at 1mM Ni^{2+} (Figs. 18 and 19).

15. DISCUSSION.

The divalent cations Cd^{2+}, Zn^{2+}, Ni^{2+} and Co^{2+} inhibited stimulated LH and PRL exocytosis in permeabilized primary pituitary cells in culture. Cd^{2+} is an important industrial pollutant and is known to have a damaging effect on several organ systems (58). The effects of Cd^{2+} on biochemical and cellular processes have been described. Cd^{2+} forms complexes with many amino acids and peptides and alters the activity of many enzyme systems in vitro and in vivo (59), (60). Cd^{2+} ions uncouple oxidative phosphorylation in rat liver mitochondria in vitro and in vivo (61). Many reportsn in the literature have been concerned with the toxic effects of Cd^{2+} through its disruption of the junctions between cells in endothelial or epithelial surfaces (62), (63).

The most commonly stated mechanism of action of the divalent cations on secretion, is their blockade of Ca^{2+} channels (54), (55). Wollheim et al. (53) studied the effects of Co^{2+} on insulin secretion in intact pancreatic islets. These authors suggested that in addition to the well-known inhibition of Ca^{2+} uptake via Ca^{2+} channels, Co^{2+} may exert its action at a step that is distal to this in the insulin release process.

Further evidence that Co^{2+} acts at an intracellular locus was put forward by Thaw and co-workers (64). Co^{2+} inhibited TRH-stimulated PRL release in intact GH3 cells with half-maximal effect observed between 0.1 and 0.3 mM Co2+, a concentration not unlike that in the present study. Although Thaw et al.(64) used an intact cell model, they demonstrated the intracellular location of Co^{2+} by fluorescence quenching of intracellularly trapped quin 2.

Speizer et al. (65) concluded from their studies that heavy metals, including Cd^{2+} and Zn^{2+} in the 10 μM range inhibit the activity of and the binding of ligands to protein kinase C. A further mechanism of action of these cations was found to be the inhibition of cAMP binding to cyclic AMP-dependent protein kinases, and the subsequent inhibition of its catalytic activity.

Knight and Sugden (52) examined the effects of the divalent cations on catecholamine exocytosis in electro-permeabilized bovine chromaffin cells. This has been the only previous report on the effect of these cations in a permeabilized system. The divalent cations Sr^{2+}, Ba^{2+}, Pb^{2+}, Mn^{2+} and La^{3+} stimulated catecholamine release. These authors found that Co^{2+} and Zn^{2+}, in concentrations up to 1 mM, had no effect on catecholamine secretion. However, high concentrations (1 mM) of Sr^{2+}, Mn^{2+}, Ca^{2+} and Ba^{2+} inhibited secretion. This difference observed for the inhibitory action of Zn^{2+} and Co^{2+} may reflect a difference between the two cell types or a difference in permeabilization methodology. Although there was insufficient evidence to be conclusive, Knight et al.(52) have suggested that these divalent

cations exert their inhibitory action at the level the involvement of several classes of protein kinase C.

The divalent cations in the present study inhibited LH and PRL release triggered by PMA/cAMP, Ba^{2+}, Mn^{2+} and Ca^{2+}. Further studies are required to characterize the intracellular site(s) of action of these cations.

In conclusion, in addition to the blockade of Ca^{2+} channels, these cations act by inhibiting exocytosis at an intracellular site which is common to diverse stimuli.

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TABLE 1.

RATIOS FOR CALCIUM SOLUTIONS AT pH 6.6

free Ca^{2+}	Ca:EGTA Ratio
8	0.0051
7	0.051
6	0.35
5.5	0.63
5	0.843
4.5	0.945

FIGURES 1 to 19.

FIGS 1A+B. TIME COURSE AND CONCENTRATION DEPENDENCE OF ∝-TOXIN INDUCED FRACTIONAL EFFLUX OF 2-DEOXY(^{3}H)GLUCOSE.

Cells were washed for 1 min in glucose-free buffer (IBF) containing 1 mM Ca^{2+} and 1 mM Mg^{2+}. Washed cells were then loaded with 1 μCi /ml of 2-deoxy (^{3}H) glucose in glucose-free buffer I (0.5 ml/well) for 40 min. Cells were then washed 7 times (3 times briefly and 4 times for 5 min on a slowly rotating shaker in buffer I, 1 ml/well). Efflux was measured at 0-10 min and at 10-20 min and is expressed below as a percentage of total cellular content as well as nett efflux after the given time periods.

FIG. 1A.

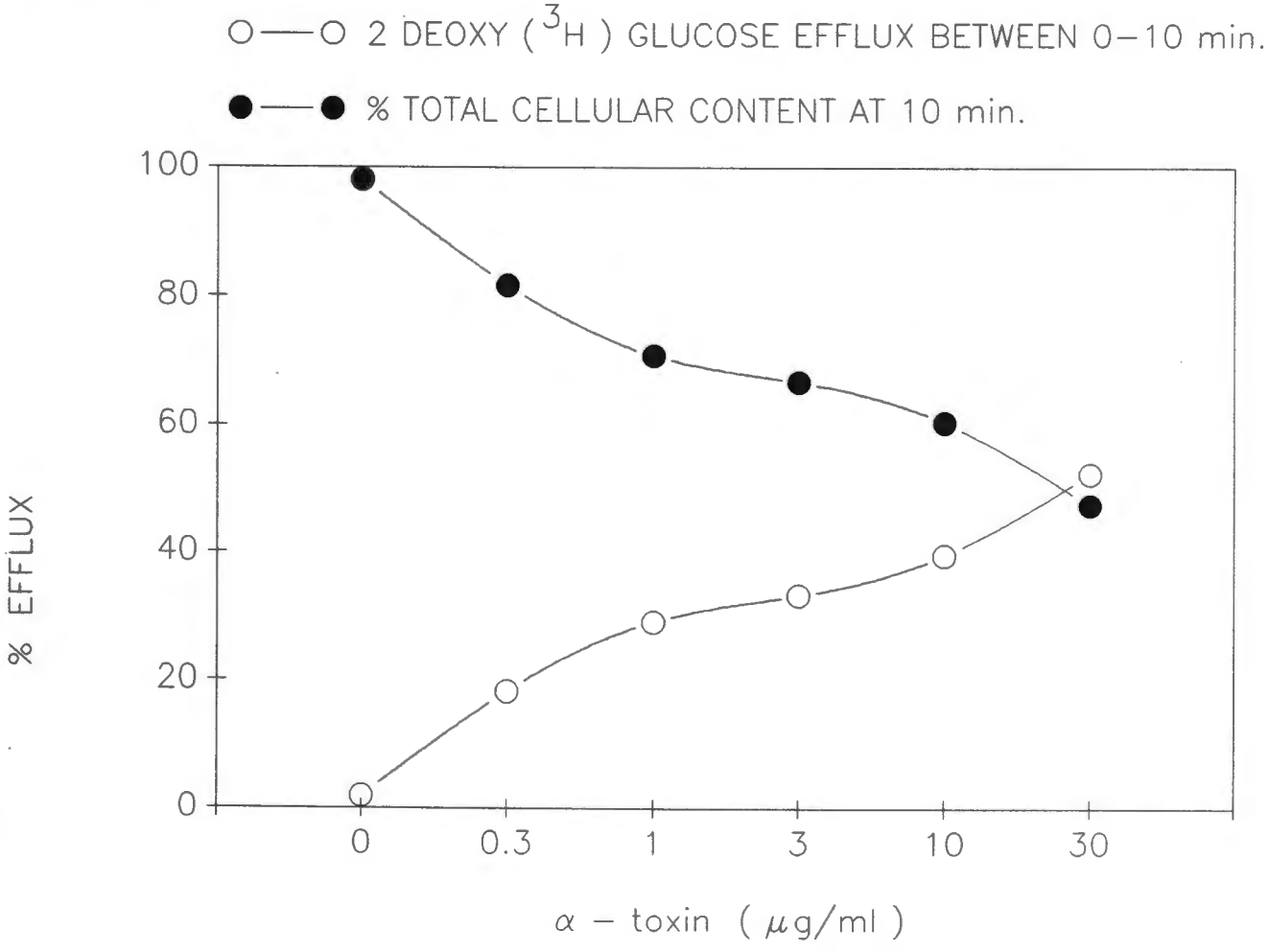

FIG. 1B.

These results are from a single experiment which was representative of three similar experiments on previous occasions.

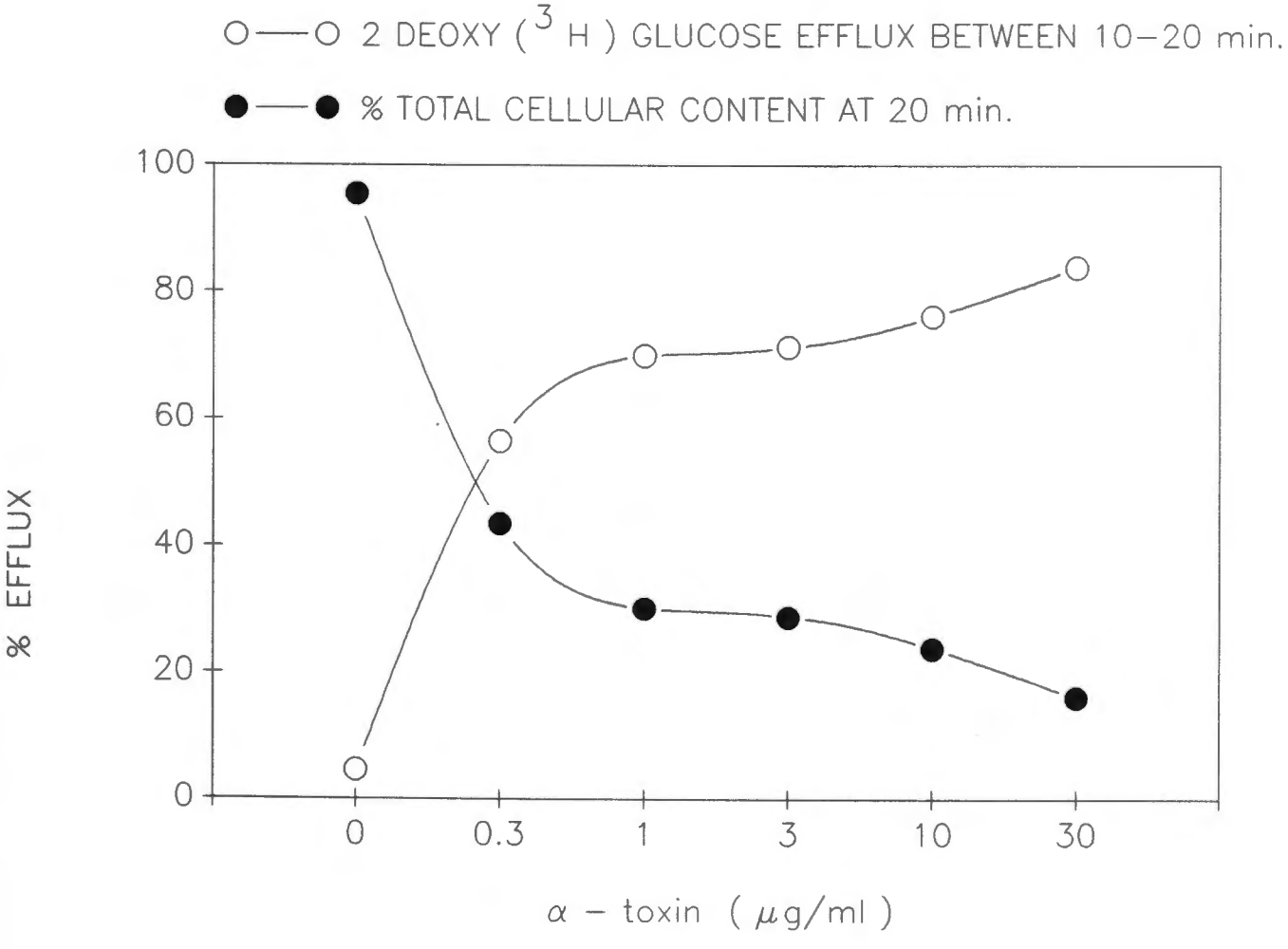

FIG.2.TIME COURSE OF LH RELEASE IN ∝-TOXIN PERMEABILIZED CELLS.

Cells were washed three times for 10 min / wash in buffer I containing 1 mM Mg^{2+} and 1 mM Ca^{2+} at 37°C. Following washing, cells were permeabilized with staphylococcal ∝-toxin for 15 min. at 37°C. LH exocytosis was initiated by incubation at 37°C with buffer IC containing free Ca^{2+} 100 nM (pCa 7), free Ca^{2+} 10 uM (pCa 5), PMA 100 nM and cAMP 30 μM as indicated. After each time period the stimulation media was aspirated and completely replaced. Therefore the graph depicts the rate of LH release as a function of time. Results are representative of similar experiments.

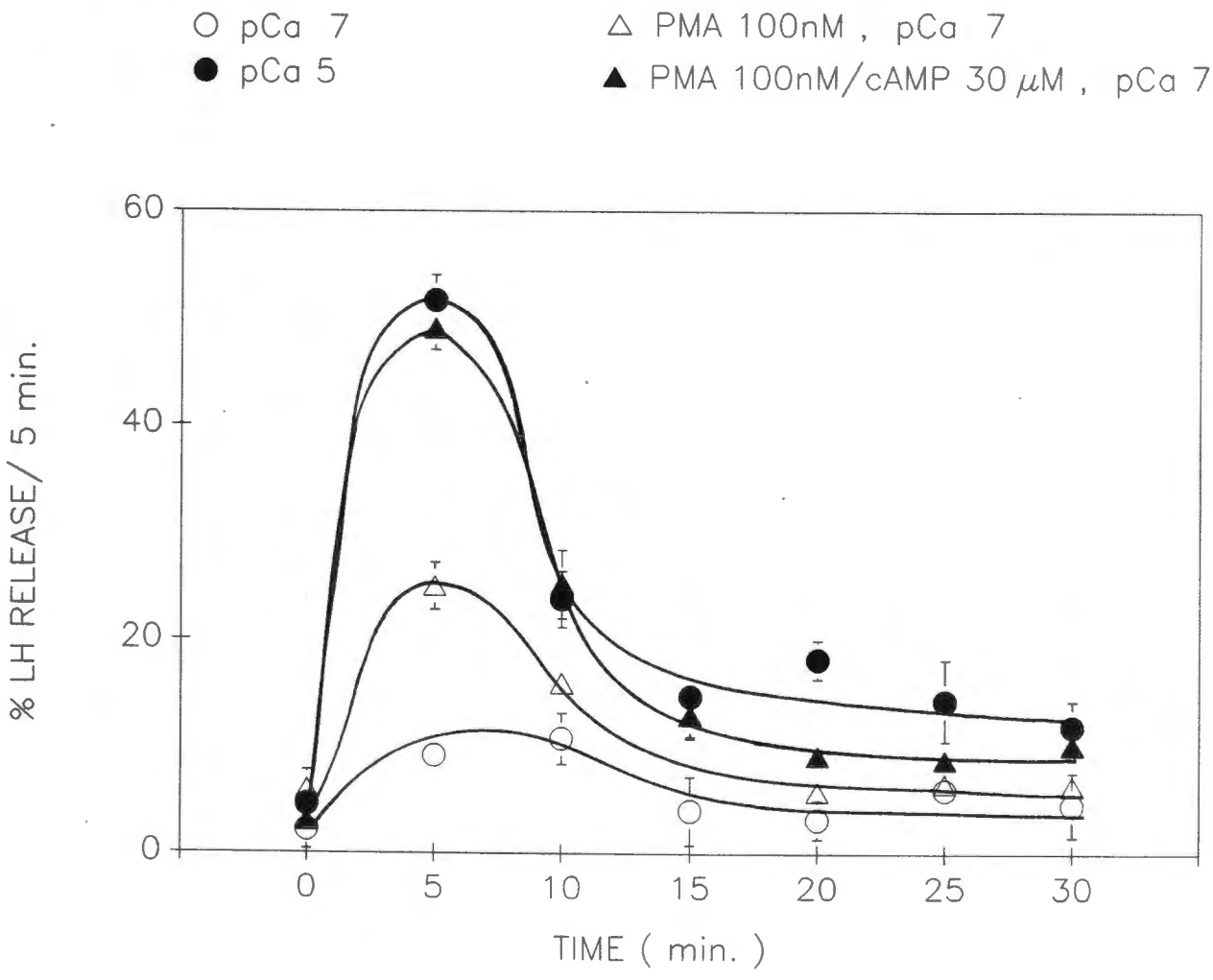

FIG. 3. EFFECTS OF FREE Ca^{2+}, cAMP AND PMA ON LH EXOCYTOSIS.

Cells were washed in buffer IC and then permeabilized as described. CaEGTA was added to the stimulation media to give the final free Ca^{2+} as indicated. PMA (100nM) and cAMP (30 μM) were added to the relevant stimulation media. Cells were stimulated for 20 min at 37°C. After this period, the media were aspirated and assayed for released LH. Results are expressed as a percentage of the total cellular LH content.These results are representative of similar previous experiments on three occasions.

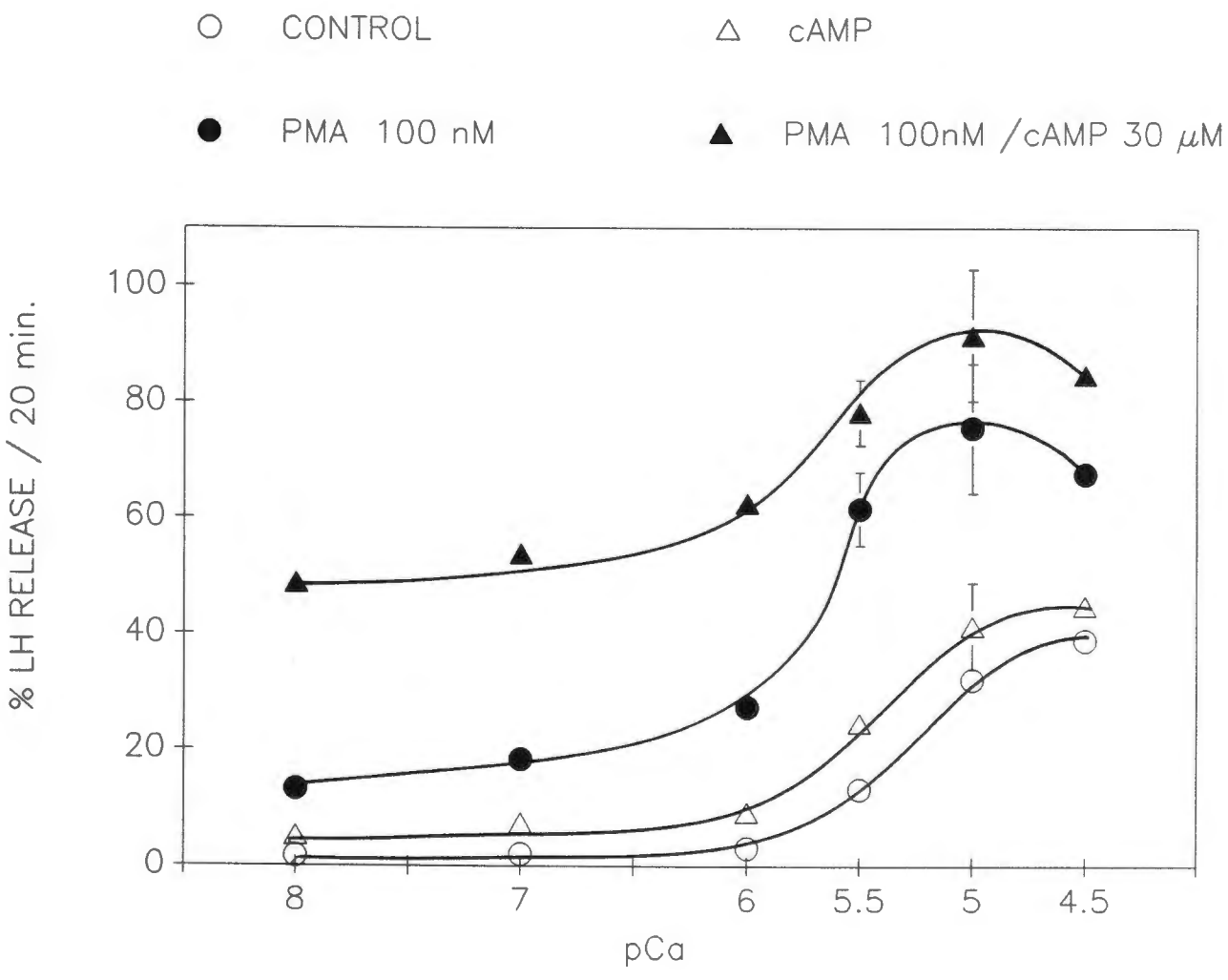

Fig. 4. cAMP DOSE-RESPONSE CURVE.

Permeabilized cells were stimulated for 20 min with buffer IC containing concentrations of cAMP ranging from 0 - 30 μM. The stimulation media contained free Ca^{2+} 100 nM (pCa 7) and IBMX 250 uM. PMA (100 nM) was either present or was not included in stimulation media as indicated. These results are representative of three similar experiments .

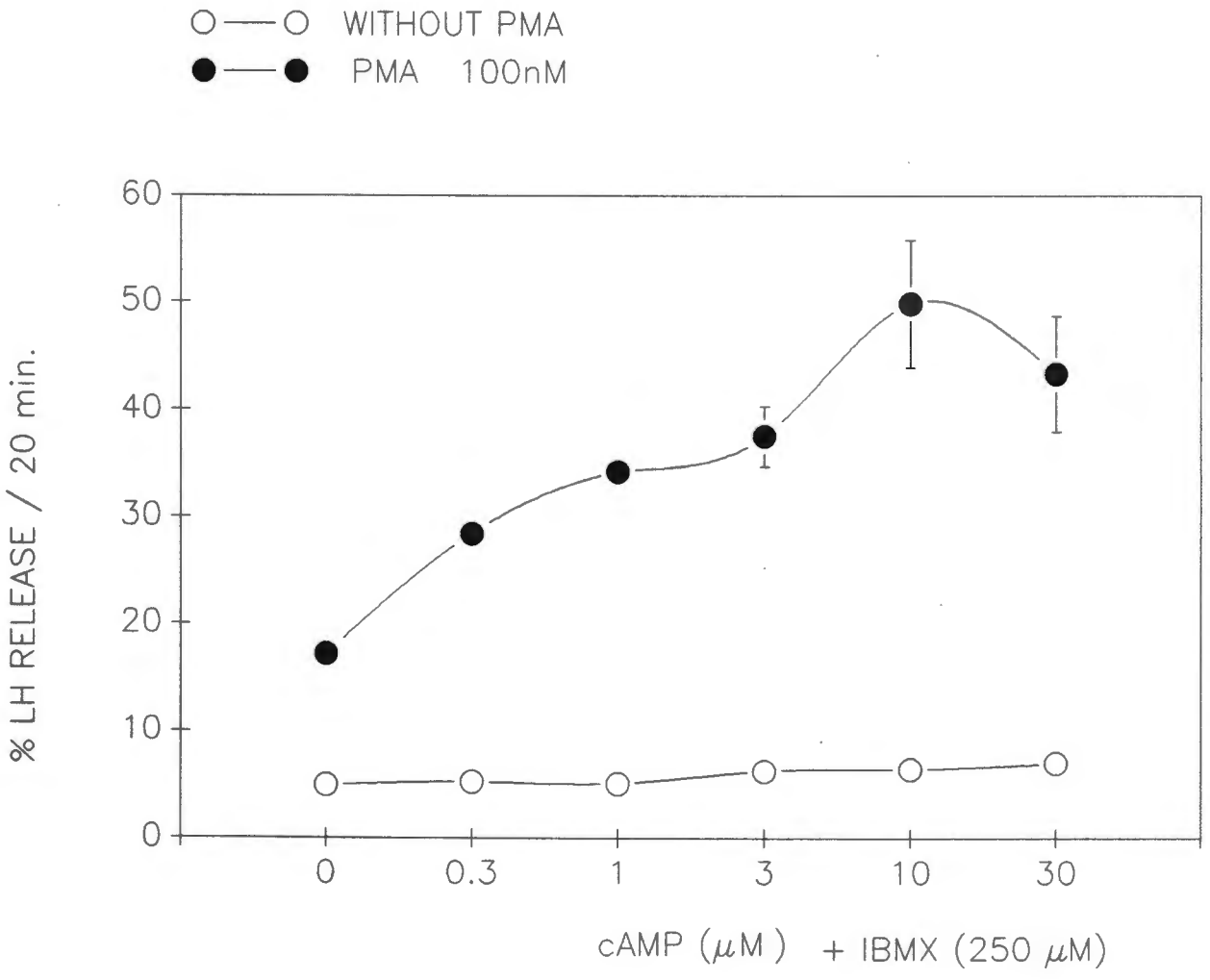

Fig. 5. EFFECT OF FREE Ca^{2+}, cAMP AND PMA ON PRL RELEASE.

Permeabilized cells were incubated at 37°C in buffer IC containing the required concentration of CaEGTA to give the indicated free Ca^{2+}. PMA (100 nM) and cAMP (30 μM) were added to stimulation media where indicated. The cells were stimulated for 20 min after which the media were aspirated and assayed for released PRL. Results are expressed as a percentage of the total cellular PRL content.

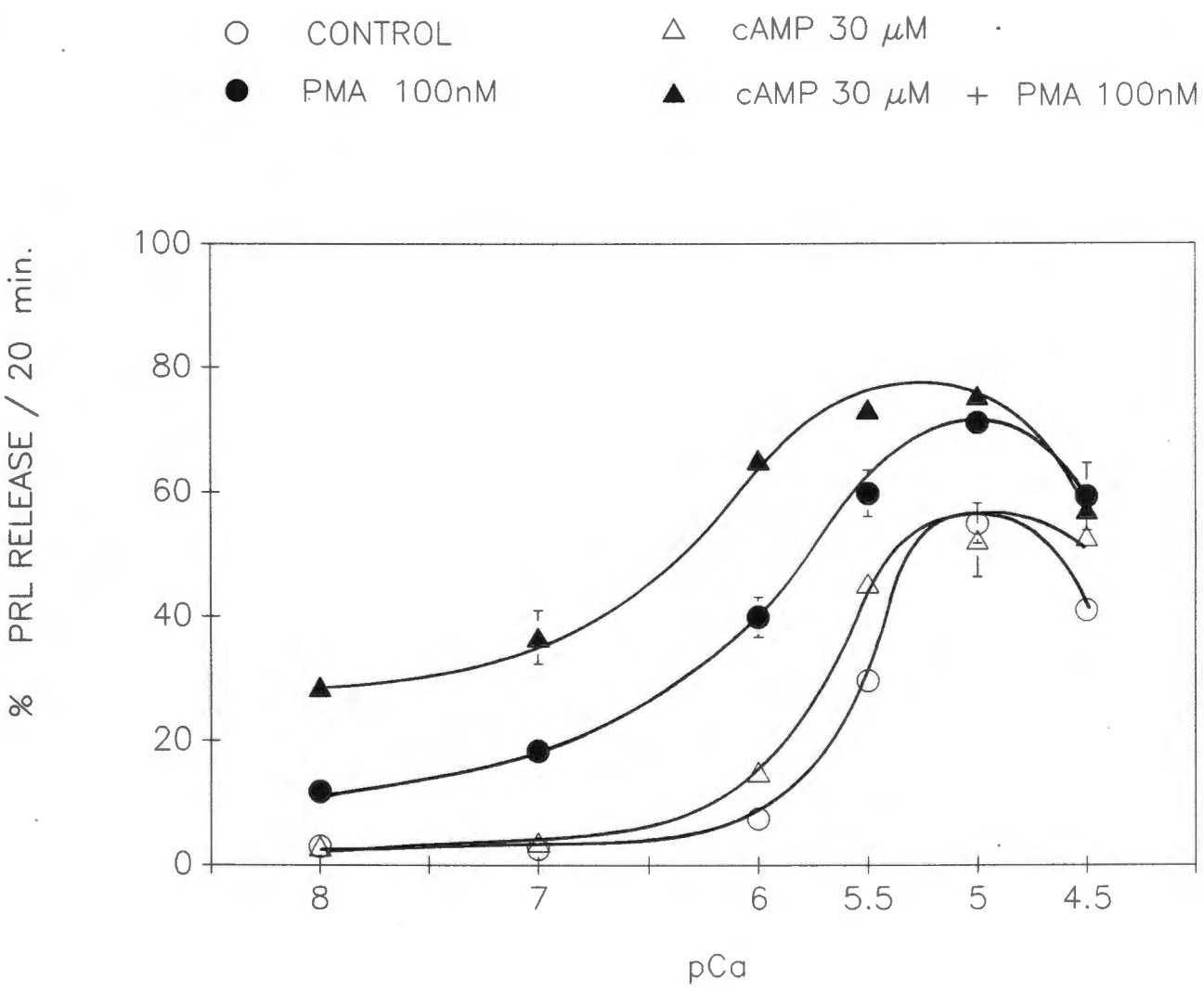

Fig. 6. EFFECT OF Ca^{2+}, cAMP AND PMA ON GH EXOCYTOSIS.

Permeabilized cells were incubated with the required concentration of CaEGTA to give the indicated free Ca^{2+}. Exocytosis was initiated by the addition of the stimulation media at 37°C. The stimulation media contained PMA (100 nM) and cAMP (30 μM) where indicated. After 20 min, the media were aspirated and assayed for released GH content. The results are expressed as a percentage of total cellular GH content.

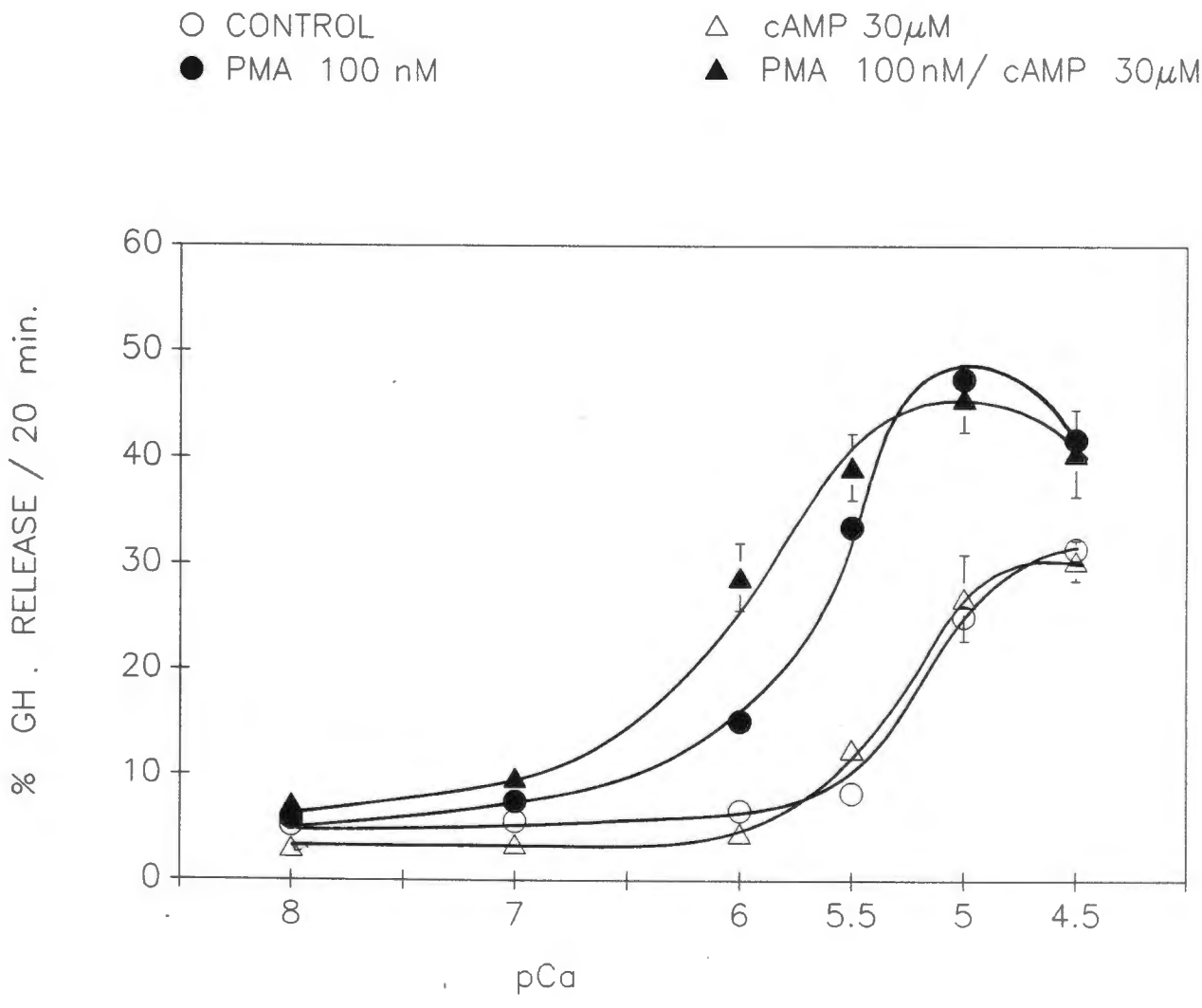

Fig. 7. Mn^{2+}-STIMULATED LH RELEASE IN PERMEABILIZED CELLS.

LH exocytosis was initiated by the addition of stimulation media at 37°C to the permeabilized cells. The stimulation media contained Mn^{2+} in a range of concentrations from 0-10mM. After 20 min the media were aspirated and assayed for released LH. The results are representataive of three similar experiments.

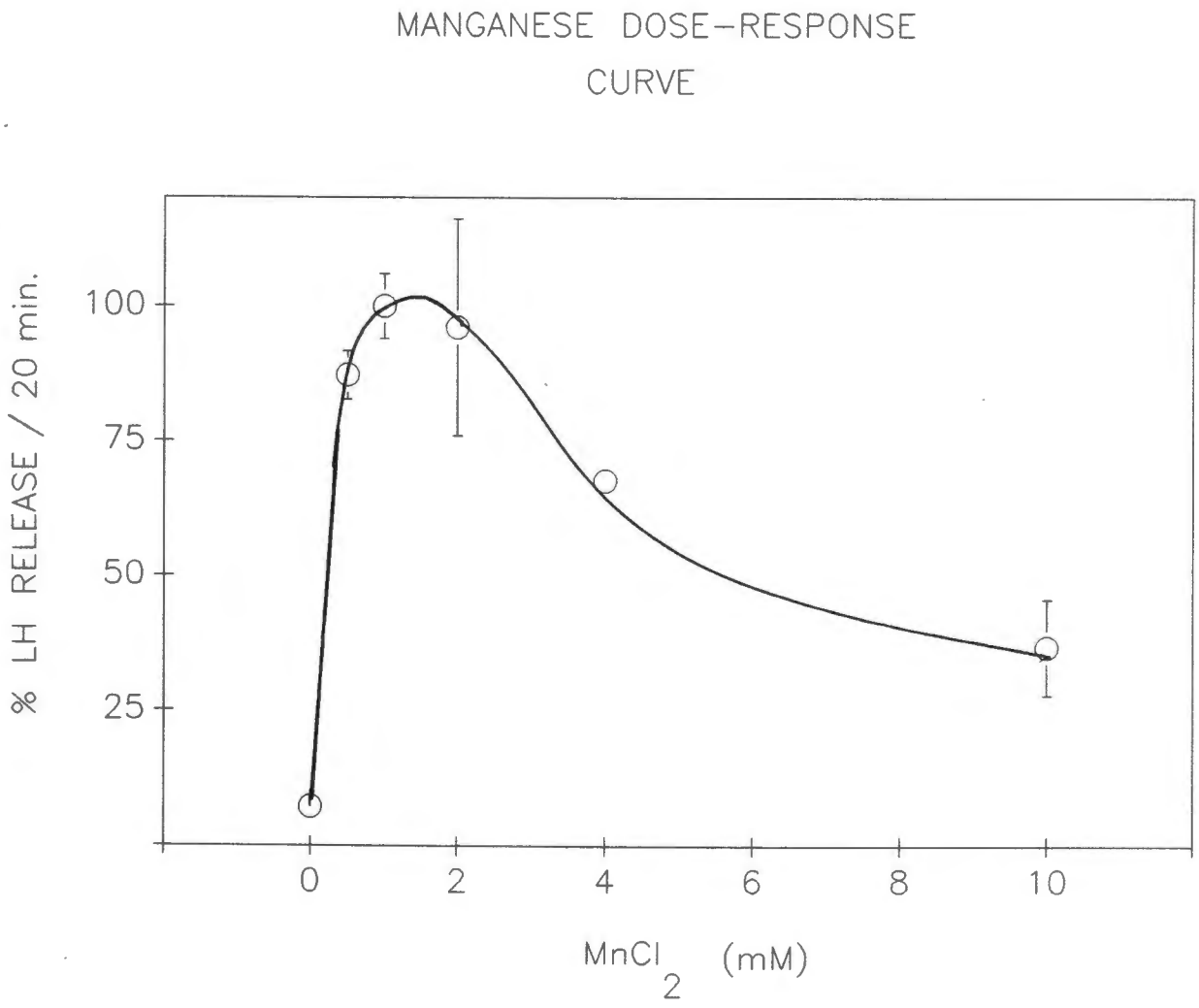

Fig. 9. THE DEPENDENCE ON ATP OF Ca^{2+}- ,PMA- AND PMA /cAMP-STIMULATED LH RELEASE.

After permeabilization cells were depleted of ATP by placing the culture plates on ice for 40 min during which time the ATP leaked out of the cells. Exocytosis was initiated by adding the stimulation media at 37°C with or without MgATP . After 20 min, the stimulation media were aspirated and assayed for released LH.

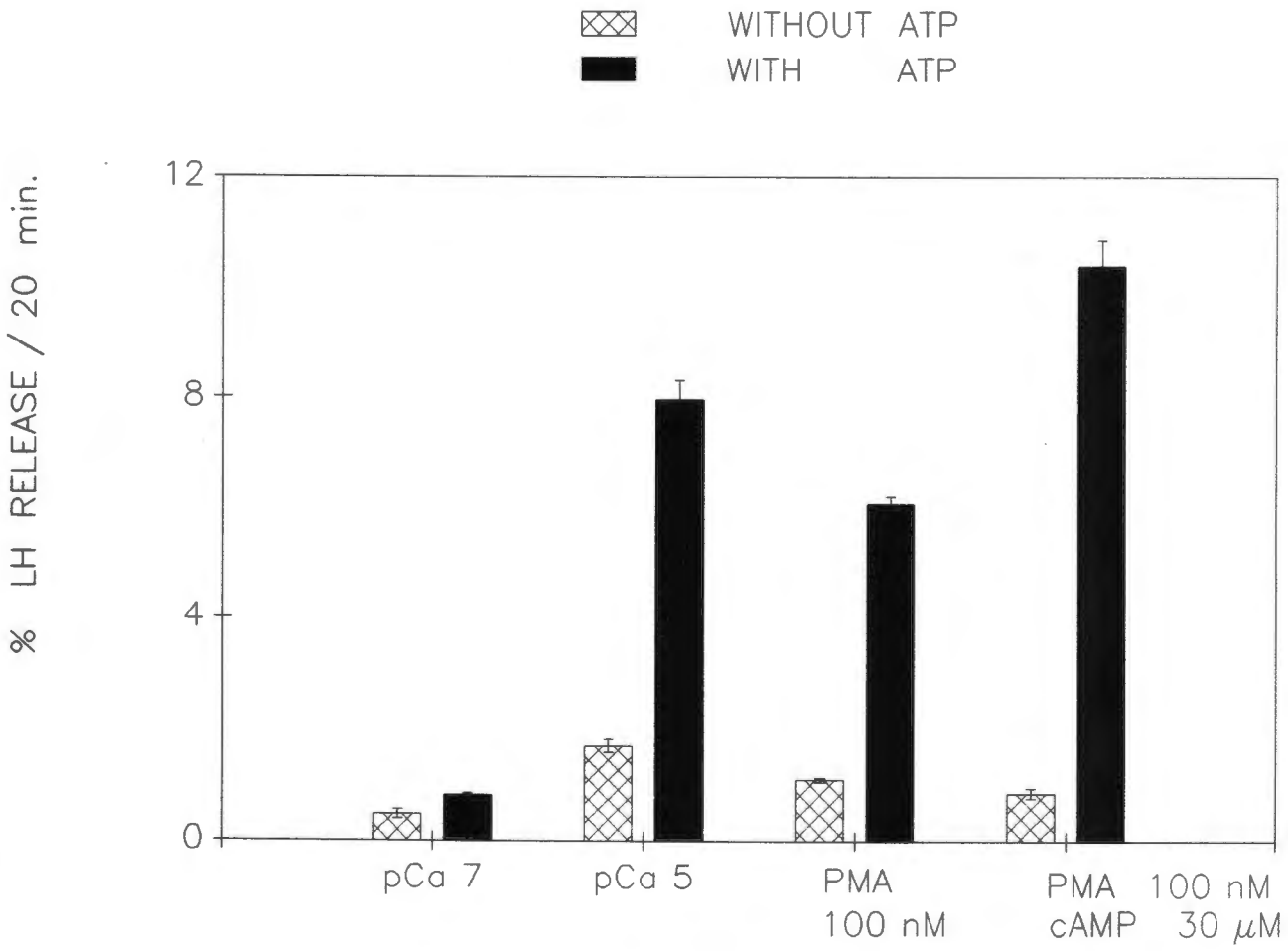

Fig. 8. DEPENDENCE OF Mn^{2+}-STIMULATED LH RELEASE ON ATP.

After permeabilization, the cells were depleted of ATP by placing the culture plates on ice for 40 min. During this time, the ATP leaked out of the cells. LH exocytosis was then initiated by adding stimulation media at 37°C to the culture plates. Cells were stimulated for 20 min, after which time the media were aspirated and then assayed for released LH. The results are representative of similar previous experiments.

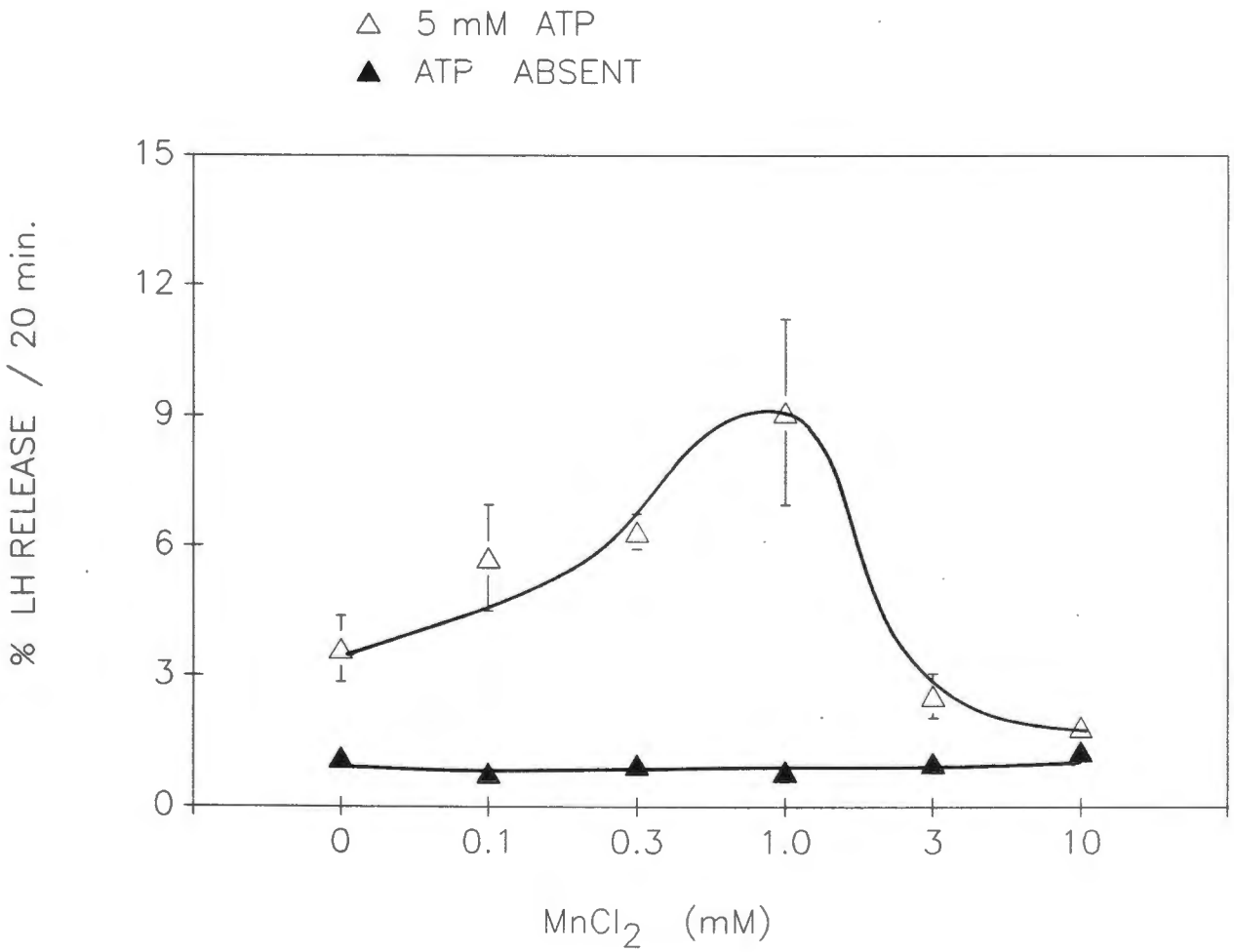

Fig. 10. EFFECT OF Mn^{2+} ON PRL EXOCYTOSIS.

Permeabilized cells were incubated with stimulation media containing the indicated concentrations of Mn^{2+} (chloride salt). After 20 min, the media were aspirated and assayed for released PRL. These results are representative of three similar experiments.

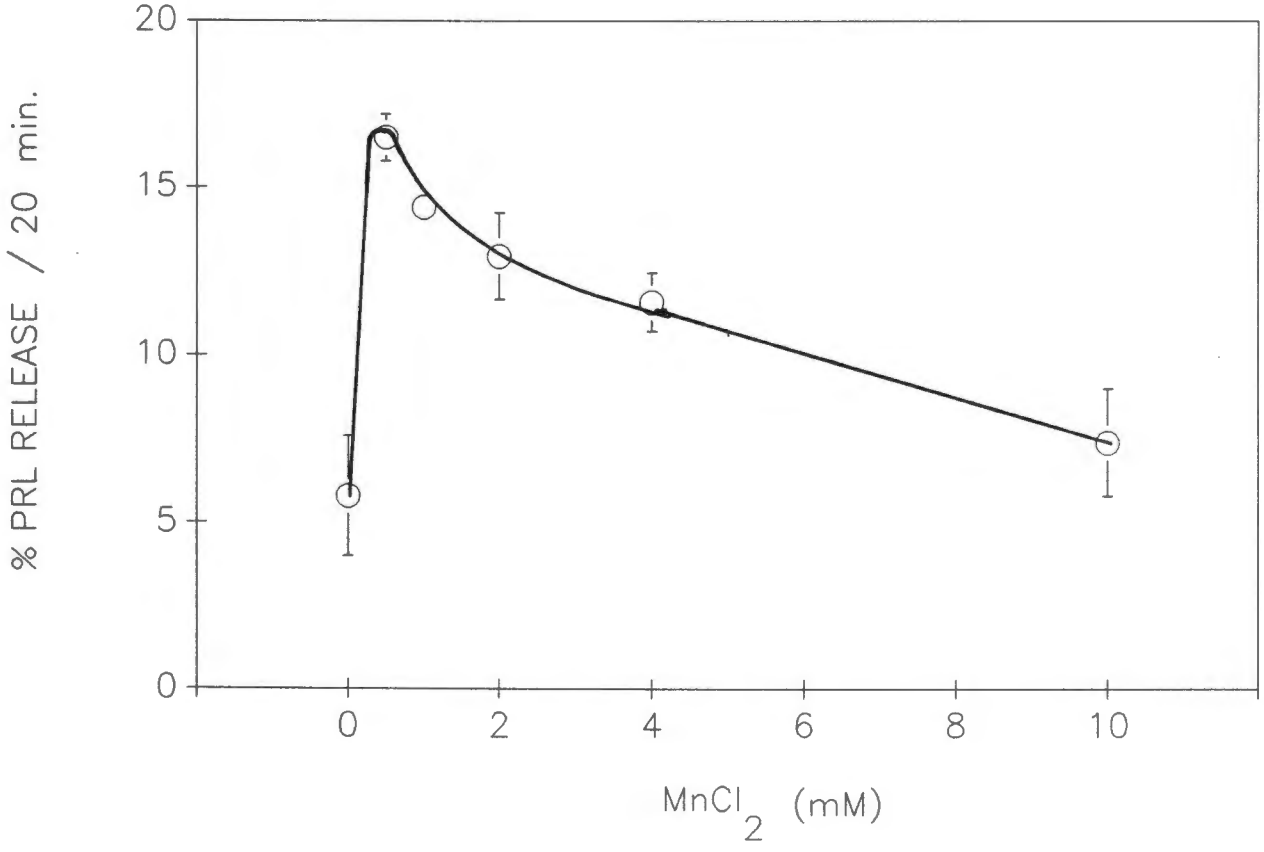

Fig. 11. DOSE RESPONSE CURVES FOR UNBUFFERED Ca^{2+} AND Ba^{2+}.

Since EGTA was not used in the stimulation media for those experiments where Zn^{2+}, Cd^{2+}, Ni^{2+} and Co^{2+} were present, the optimal stimulatory concentration of Ca^{2+} and Ba^{2+} in the unbuffered system had to first be determined. Cells were washed twice with buffer I containing $CaCl_2$ (1mM) and once with Ca^{2+}-free buffer I. $CaCl_2$ and $BaCl_2$ were added to the stimulation media in a range of concentrations ranging from 0 to 10 mM. The percentage of released LH was determined after 20 min stimulation.

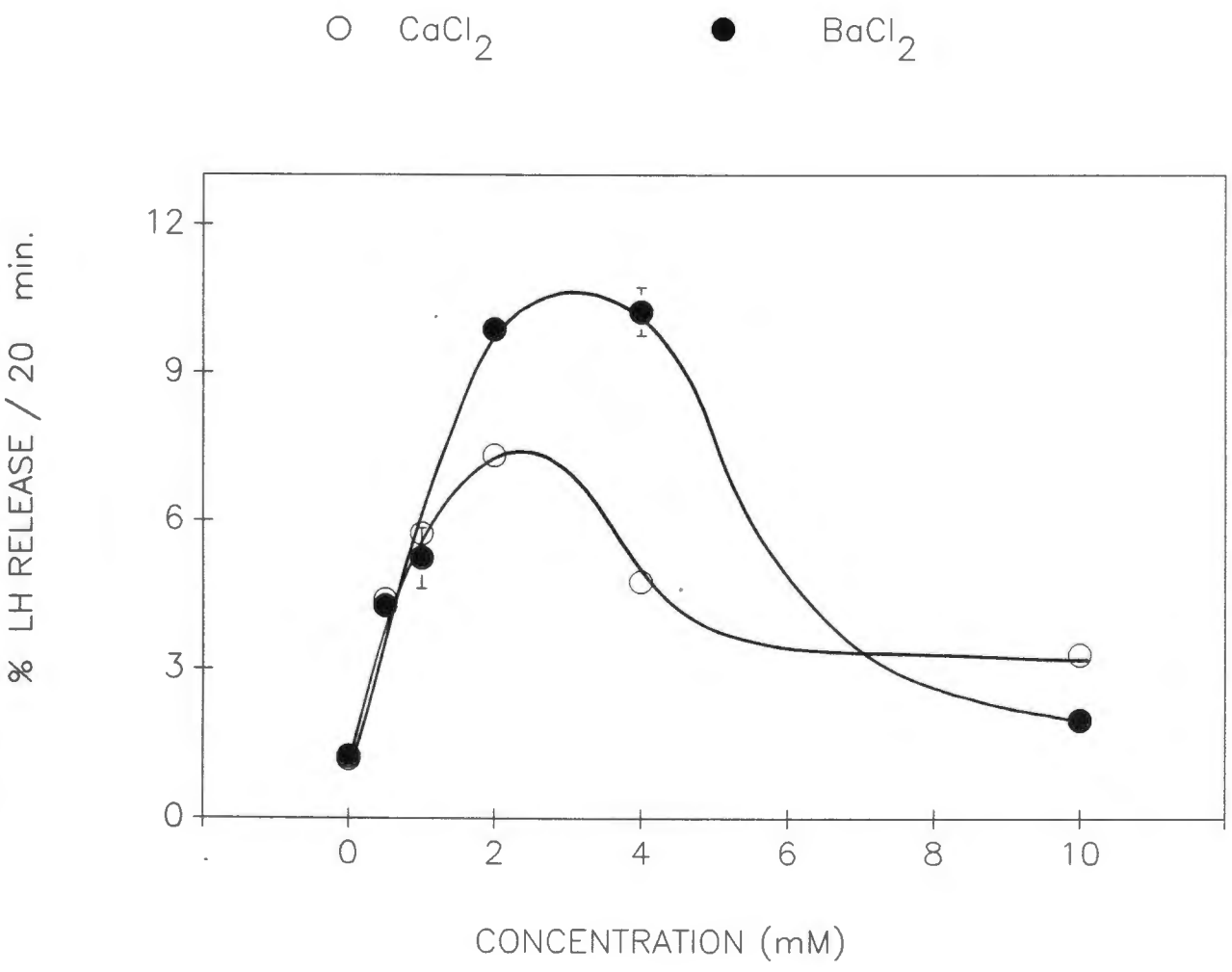

Fig. 12. INHIBITORY EFFECT OF Cd^{2+} ON STIMULATED LH EXOCYTOSIS.

Cells were washed twice with buffer I containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and once in Ca^{2+}-free buffer I. Cd^{2+} (chloride salt) was added to the permeabilization media in the indicated concentrations. The stimulation media contained $CaCl_2$, 2mM; $BaCl_2$, 2 mM; Mn^{2+}, 2 mM; cAMP, 30 μM; and PMA, 100 nM as indicated. $CdCl_2$ was also present in the stimulation media at the indicated concentrations. The results have been normalized to percentage of maximal stimulated LH secretion without Cd^{2+} present. These results are representative of similar previous experiments.

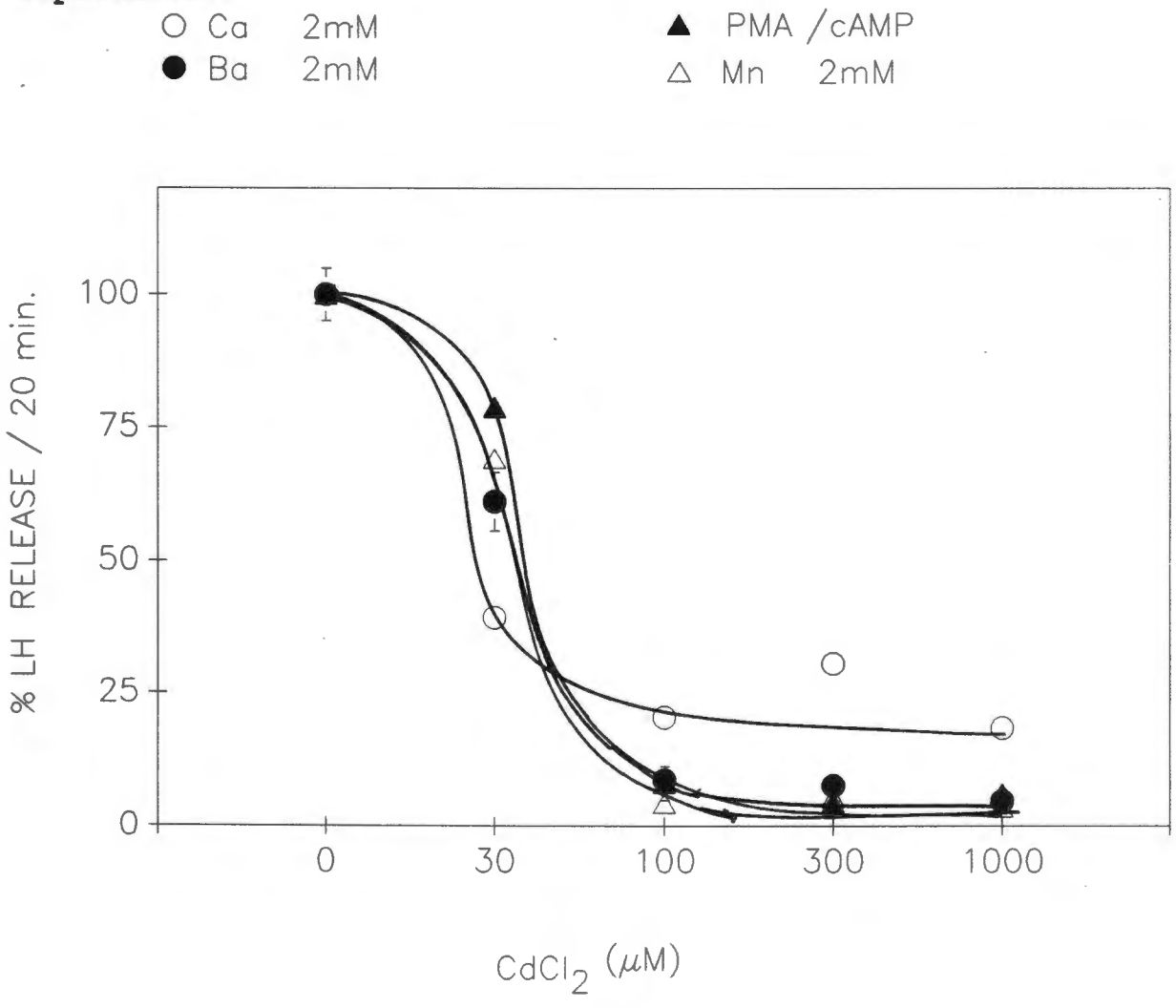

Fig. 13. EFFECT OF Cd^{2+} ON STIMULATED PRL RELEASE.

Cells were washed twice with buffer I containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and once in Ca^{2+}-free buffer I. Cd^{2+} (chloride salt) was added to the permeabilization media and to the stimulation media as indicated . $CaCl_2$, 2 mM; $BaCl_2$, 2 mM; $MnCl_2$, 2mM; cAMP, 30 μM and PMA 100 nM were added to the stimulation media where indicated. The results have been normalized to the percentage of maximal stimulated PRL secretion without Cd^{2+} present. These results are representative of similar previous experiments.

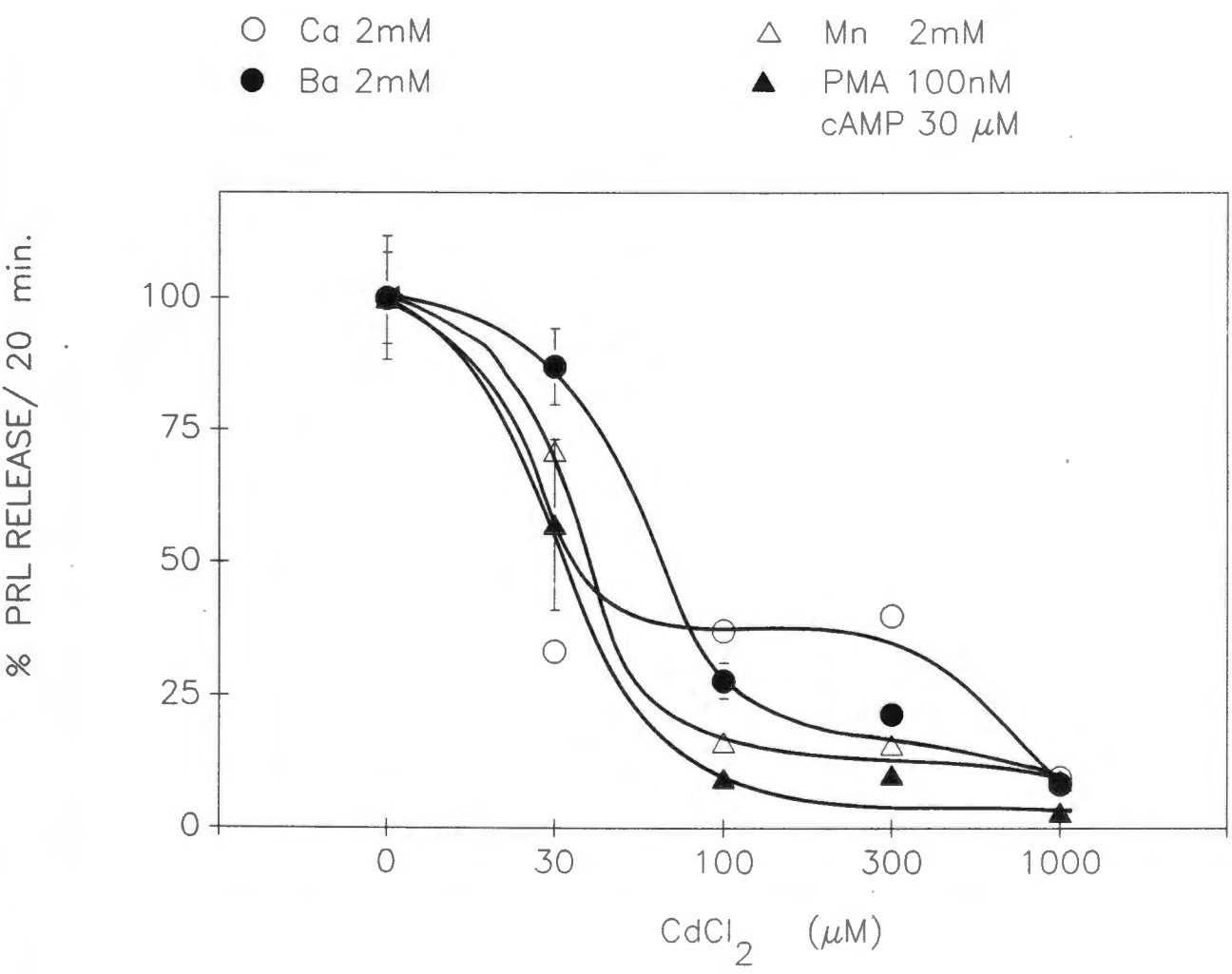

Fig. 14. THE EFFECT OF Zn^{2+} ON STIMULATED LH SECRETION.

Cells were washed twice with buffer I containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and once in Ca^{2+}-free buffer. Zn^{2+} (chloride salt) was present in the permeabilization and in th stimulation media in the indicated concentrations. $CaCl_2$, 2mm; $MnCl_2$, 2mM; PMA 100 nM and cAMP 30 M were added to the stimulation media where indicated. The data has been normalized as previously described. These results are representative of similar previous experiments.

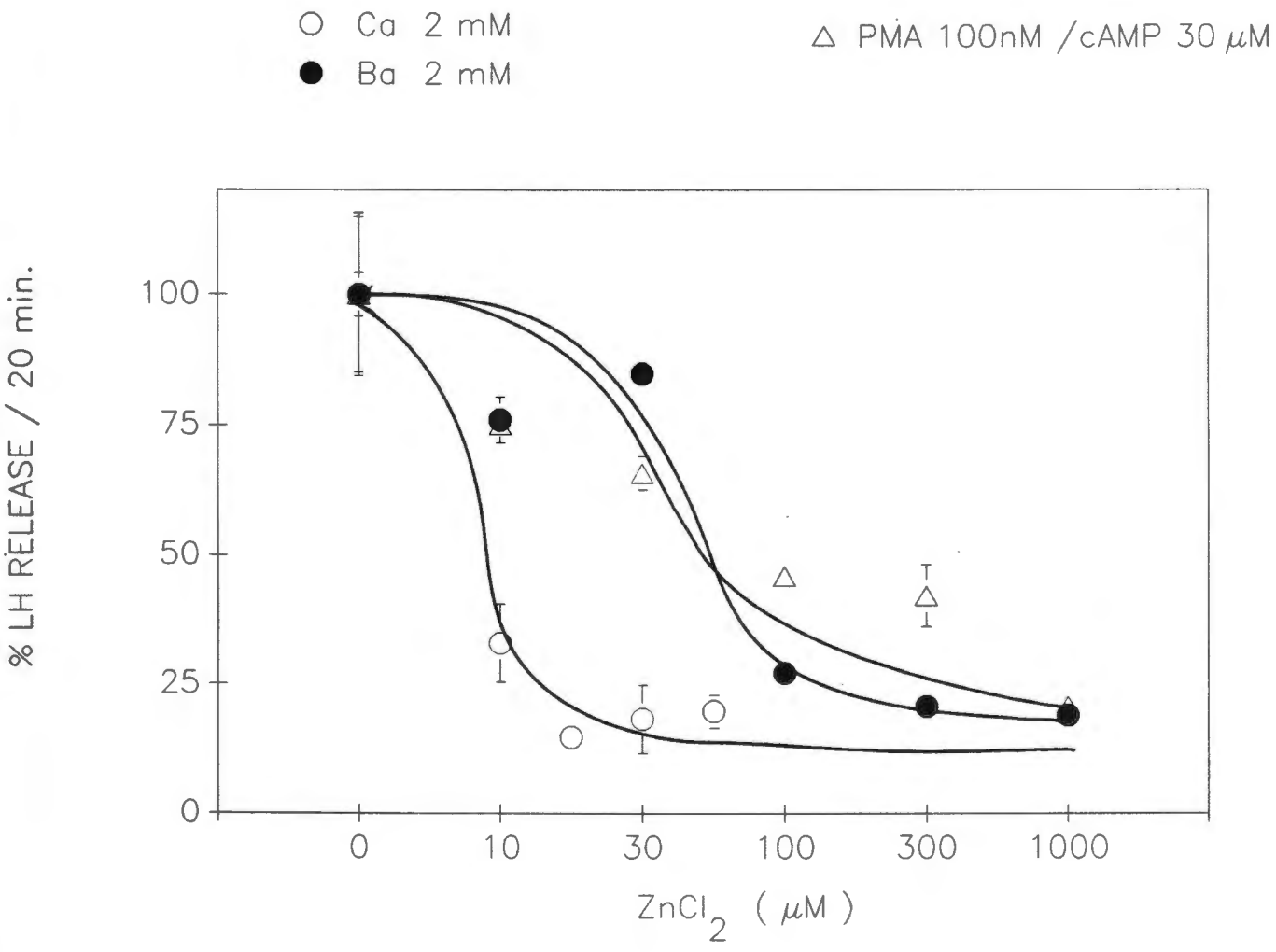

Fig. 15. EFFECT OF Zn^{2+} ON STIMULATED PRL RELEASE.

Cells were washed twice with buffer I as previously. The final wash was with Ca^{2+}- free buffer I. Zn^{2+} (chloride salt) was present in the permeabilization media as well as in the stimulation media in the indicated concentrations. $CaCl_2$, 2 mM; $MnCl_2$ 2 mM; cAMP, 30 μM and PMA 100 nM were present as indicated in the stimulation media. The data has been normalized to maximal stimulation in the absence of Zn^{2+}. These results are representative of similar previous experiments.

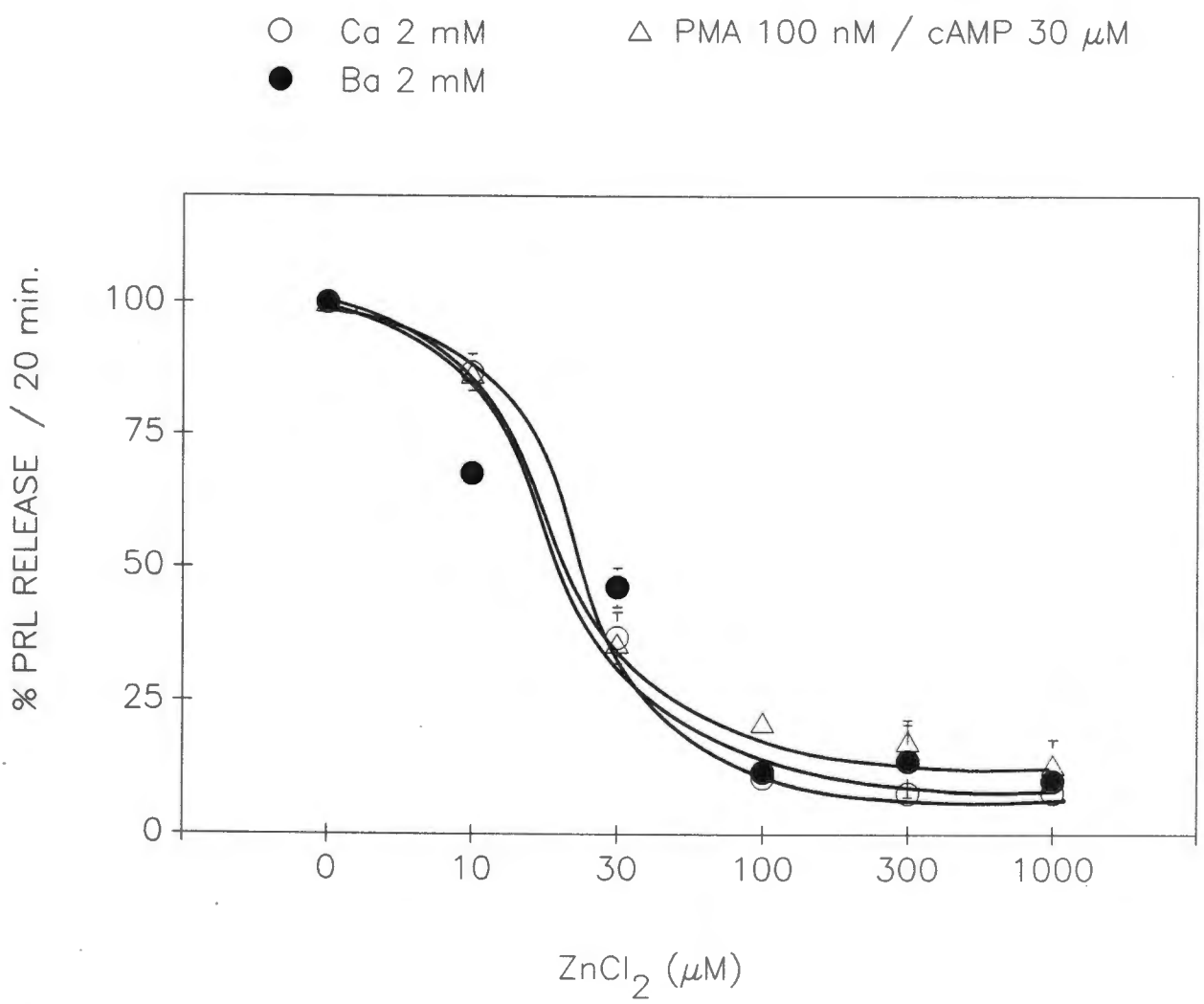

Fig. 16 EFFECT OF Co^{2+} ON STIMULATED LH RELEASE.

Co^{2+} (chloride salt) was added to both the permeabilization and the stimulation media in the indicated concentrations. $MnCl_2$ 2 mM; $CaCl_2$ 2 mM; $BaCl_2$, 2 mM; cAMP 30 μM and PMA 100 nM were added to the stimulation media as indicated. The results have been normalized to maximal stimulation without Co^{2+} present. These results are representative of similar previous experiments.

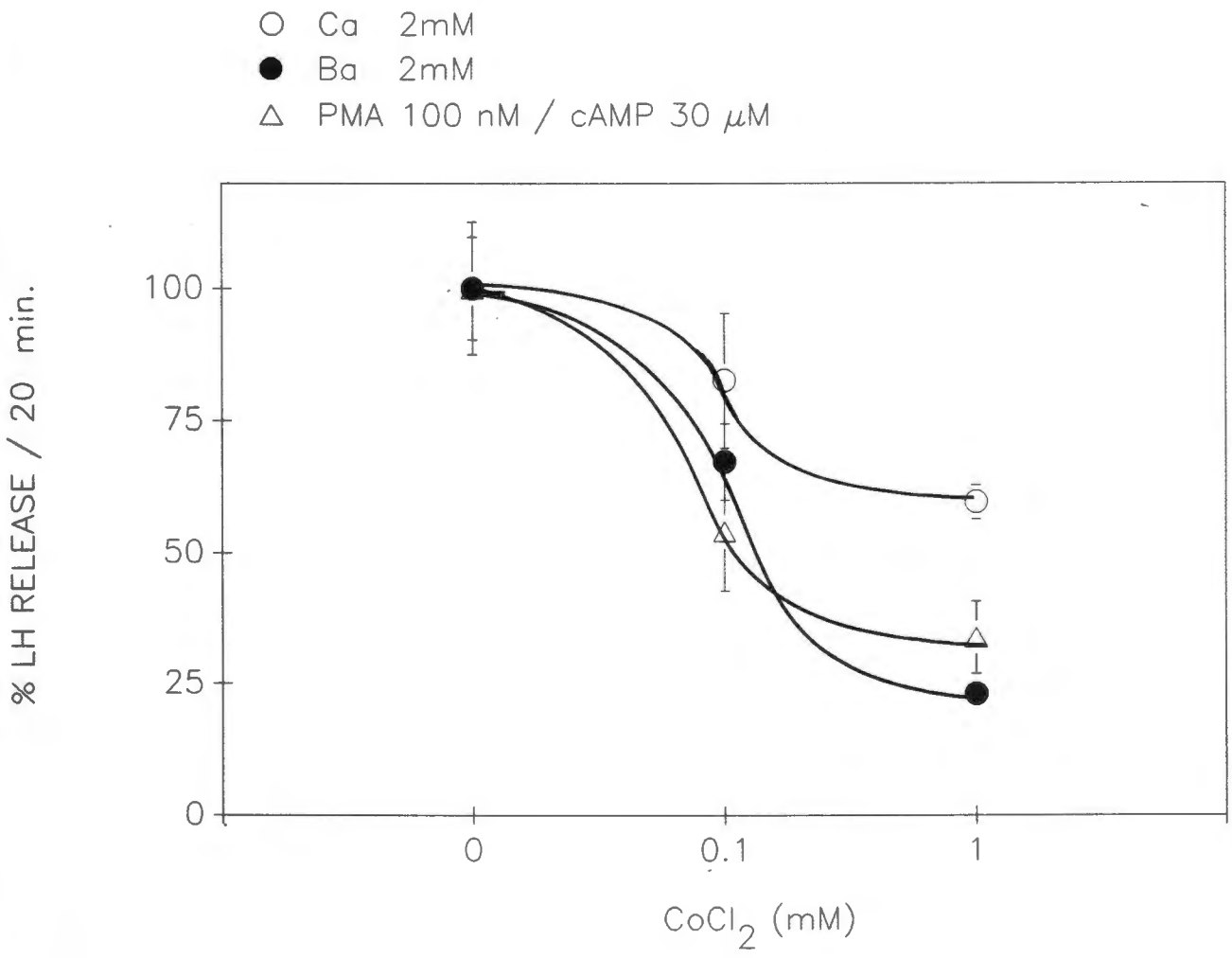

Fig 17. THE EFFECT OF Co^{2+} ON STIMULATED PRL RELEASE.

$CoCl_2$ was added to both the permeabilization and stimulation media as indicated. $CaCl_2$, 2 mM; $BaCl_2$, 2 mM; $MnCl_2$, 2 mM; cAMP 30 μM and PMA 100 nM were added to the stimulation media where indicated. The data has been normalized to the maximal stimulation in the absence of Co^{2+}. These results are representative of similar previous experiments.

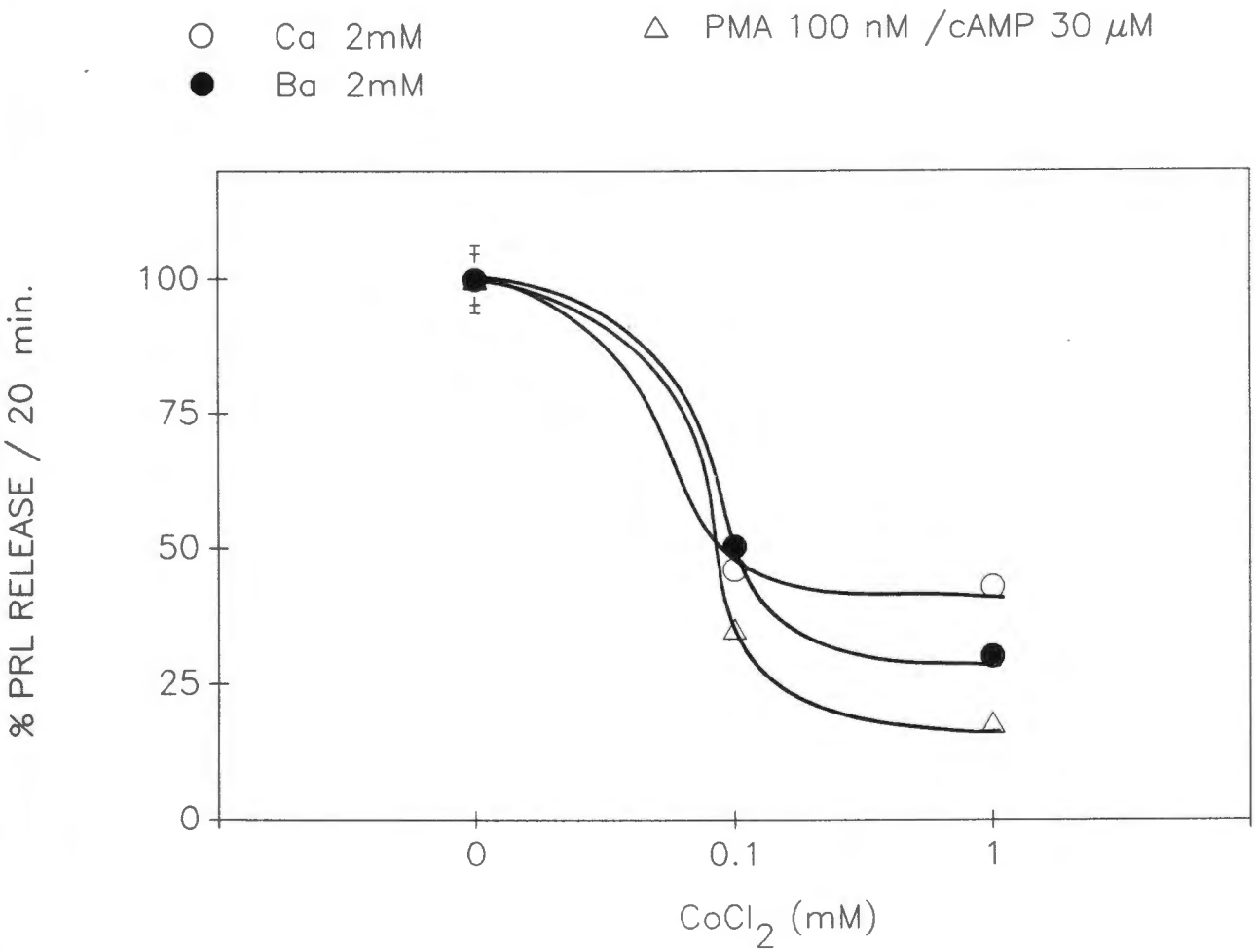

Fig. 18. THE EFFECT OF Ni^{2+} ON STIMULATED LH EXOCYTOSIS.

$NiCl_2$ was added to both the permeabilization and to the stimulation media as indicated. $CaCl_2$, 2 mM; $MnCl_2$, 2 mM; $BaCl_2$, 2 mM; cAMP, 30 μM and PMA 100 nM were added to the stimulation media where indicated. The results have been normalized to the maximal release without Ni^{2+} present. These results are representative of previous experiments.

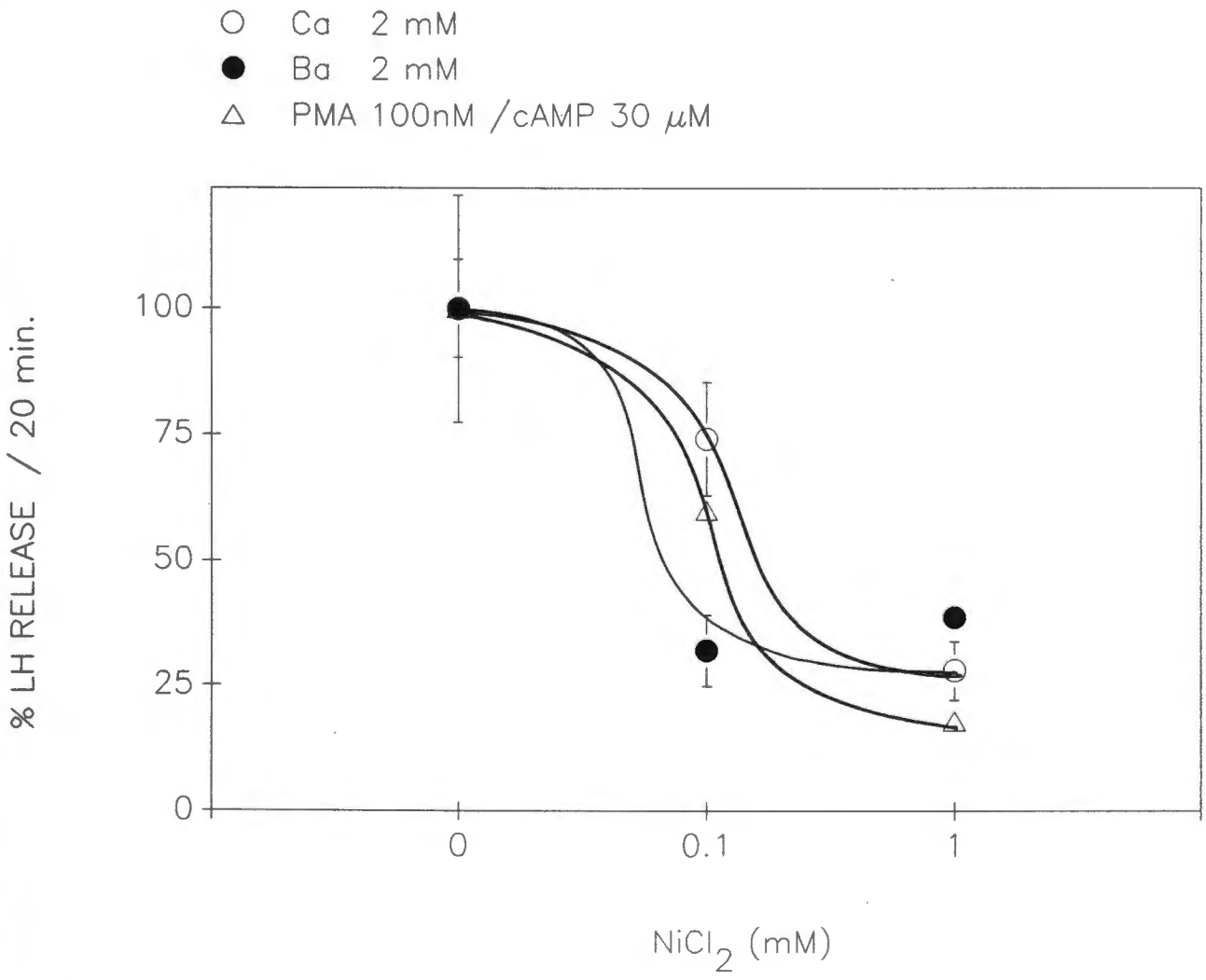

Fig. 19. EFFECT OF Ni^{2+} ON STIMULATED PRL EXOCYTOSIS.

$NiCl_2$ was added to both the permeabilization and stimulation media in the indicated concentrations. $CaCl_2$, 2 mM; $BaCl_2$, 2mM; $MnCl_2$, 2mM; cAMP 30 μM and PMA 100 nM were added to the stimulation media where indicated. The results have been normalized to the maximal stimulation in the absence of Ni^{2+}. The results are representative of similar previous experiments.